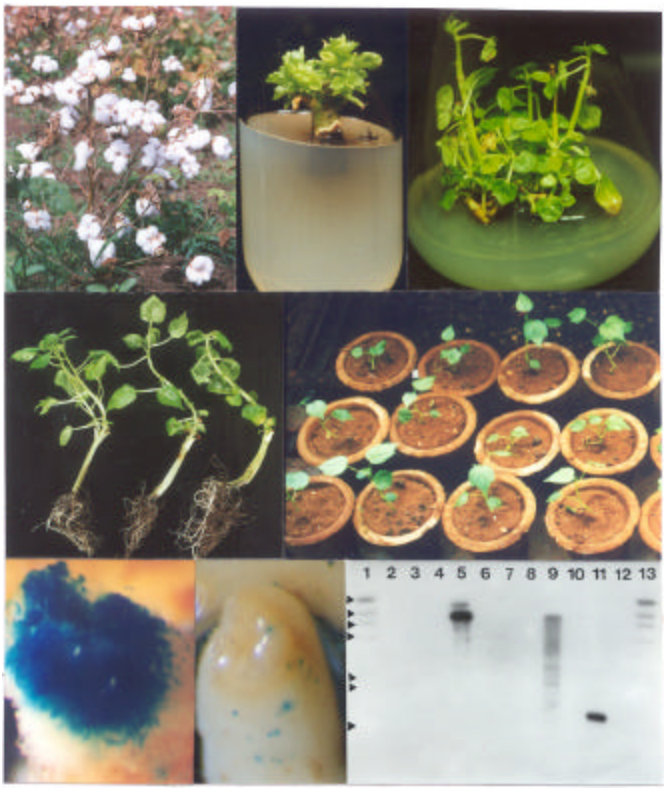
IN VITRO REGENERATION AND GENETIC TRANSFORMATION STUDIES IN INDIAN CULTIVARS OF COTTON

(*Gossypium hirsutum L.*)



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JANUARY 2001

IN VITRO REGENERATION AND GENETIC TRANSFORMATION STUDIES IN INDIAN CULTIVARS OF COTTON (*Gossypium hirsutum L.*)

A THESIS

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BY

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Dedicated

to my

Maa, Mama & Dada

CONTENTS

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Date:
Pune: *(A. K. Banerjee)*

CERTIFICATE

This is to certify that the work incorporated in the thesis entitle **" *In vitro* regeneration and genetic transformation studies in Indian cultivars of cotton (*Gossypium hirsutum* L.)** submitted by Mr. A. K. Banerjee 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)
Guide

Pune

Date:

Key to abbreviations

B5	Gamborg's medium (1968)
BAP	6-Benzyl amino purine
bp	Base pairs
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivars
^0C	Degree Celsius
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
GA_3	Gibberellic acid
Kb	Kilobases
KIN	Kinetin (6-furfuryl amino purine)
MS	Murashige and Skoog medium (1962)
NAA	α-Naphthaleneacetic acid
PDS	Particle Delivery System
PVP	Polyvinyl pyrrolidone
q/ha	Quintal per hectare
SDS	Sodium dodecyl sulphate
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea
Vol/Vol	Volume/volume (concentration)
Wt/Vol	Weight/ volume (concentration)

SYNOPSIS

SYNOPSIS

The Genus *Gossypium* belonging to the family Malvaceae includes fifty species of which only four are domesticated (Fryxell 1992). Among these four, two species (*Gossypium arboreum* L. and *G. herbaceum* L.) are native to the old world and are diploids (2n = 26), whereas the other two species (*G.hirsutum* L. and *G. barbadense* L.) are domesticated in the new world and are tetraploids (2n = 52). The diploid species contribute 2% of the total world cotton production whereas 90% cotton grown worldwide is *G. hirsutum* and remaining 8 % accounts by *G. barbadense* (Lee 1984).

Cotton is one of the most important commercial crop of the world valued for its fiber, oil and other by products. It is grown in 70 countries and over 180 million people around the globe are involved with the fiber industry which produces 20 - 30 billion US dollars of raw cotton (John 1997).

Cotton crop is susceptible to a wide range of insect pests, mainly lepidopteran insects. The crop loss due to insects alone has been estimated to be over 600 million US $ per year. Conventional plant breeding techniques have limitations to solve this problem (Pannetier et al. 1997). Recently, major efforts have been directed towards the introduction of new agricultural traits through genetic engineering. Insect resistance is one the most important goal for the cotton improvement. Insecticidal protein genes of *Bacillus thuringiensis* var. *kurstaki Cry IA(b)* and *CryIA(c)* have been incorporated into cotton cultivar Coker-312 via *Agrobacterium tumefaciens* mediated transformation (Perlak et al. 1990). However, majority of these reports pertain to Coker cultivars which are not cultivated in India. Since India occupies a position among the top five cotton producing countries in the world, concerted efforts are being made to develop transgenic cotton varieties of our local cultivars.

The present work entitled **"*In vitro* regeneration and genetic transformation studies in Indian cultivars of cotton (*Gossypium hirsutum* L.)"** was undertaken with an objective to develop an efficient *in vitro* plant regeneration protocol which is a major pre-requisite for plant transformation studies. Yet another objective of the work was to study genetic transformation in cotton by *Agrobacterium* and particle bombardment methods.

The thesis has been divided into six chapters followed by a summary and a list of references and author's publications and patents.

CHAPTER 1: GENERAL INTRODUCTION

This chapter covers the introduction of the genus cotton (*Gossypium hirsutum* L) and a thorough literature survey on *in vitro* regeneration and genetic transformation studies in cotton. An insight of cotton scenario in terms of its production, area under cultivation has also been described in this chapter. Objectives and aims of the present work are also envisaged in this chapter.

CHAPTER 2: MATERIALS AND METHODS

Different methodologies employed in tissue culture, histology and genetic transformation during the course of work have been described in this chapter.

CHAPTER 3: *IN VITRO* INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION FROM COTYLEDONARY NODE AND ZYGOTIC EMBRYO AXIS EXPLANTS OF COTTON

Methods for high frequency seed germination and morphogenetic potential of explants like cotyledonary node and zygotic embryo axis of cotton have been evaluated in this chapter. This chapter also describes the influence of cytokinins and other phytohormones in induction and proliferation of multiple shoots from cotyledonary node and zygotic embryo axis explants. Histology of cotyledonary node showing multiple shoot induction has been presented. Conditions for the elongation of shoots, *in vitro* rooting and hardening of plants were also described in this chapter.

CHAPTER 4: EFFECT OF ANTIBIOTICS ON SHOOT GROWTH OF ZYGOTIC EMBRYO AXIS EXPLANTS OF COTTON

The effect of different antibiotics on high frequency shoot formation and growth from longitudinally split embryo axes have been described in this chapter.

CHAPTER 5: *AGROBACTERIUM TUMEFACIENS* MEDIATED TRANSFORMATION STUDIES IN COTTON

The different conditions of *Agrobacterium* treatment have been described. The integration of GUS (β-glucuronidase) and NPT II (neomycin phosphotransferase II) gene in callus tissue has been confirmed by histochemical assay and Southern methods respectively. Putative transformants were analysed by Southern method. Optimization of *in vitro* micrografting method was also described using control plants and putative transformants.

CHAPTER 6: TRANSIENT GENE EXPRESSION IN COTTON *VIA* PARTICLE BOMBARDMENT METHOD

Transfer of plasmids p35SGUSINT and pIBGUSINT from *Agrobacterium* strains to *E.coli* by electroporation method has been described in this chapter. Histology of plumular axis has been depicted This chapter also deals with the effect of different parameters of particle bombardment like microcarrier, rupture disks, target cell distance etc. on the expression of GUS gene in embryonic axes.

SUMMARY:

This part of the thesis summarizes the main findings of the work and its future applications.

KEY REFERENCES

1. McCabe, D.E. and Martinell, B.J. (1993) Transformation of elite cotton cultivars via particle bombardment of meristems. Bio/Technology 11: 596 -598.

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(Dr. K. V. Krishnamurthy) (A. K. Banerjee)

Research Guide Candidate

CHAPTER 1

GENERAL INTRODUCTION

Introduction

1. The crop

Cotton is one of the most important commercial crops of the world valued for its fibre, oil and other by-products. It belongs to the genus *Gossypium* under the family Malvaceae. The genus comprises 50 species, only four of which are cultivated. Out of these four species, *Gossypium arboreum* L. *and G. herbaceum* L. are diploids (2n=26), and are called Old world cotton while the other two species *Gossypium hirsutum* L. *and G barbadense* L. are tetraploids (2n=52) and are known as New world cotton. The diploid species contribute 2% of the total world cotton production whereas 90% cotton grown worldwide is *G. hirsutum* L. and the remaining 8% accounts for *G. barbadense* L. (Lee 1984). Cotton is grown in 70 countries and about 180 million people around the globe are involved with the fiber industry which produces raw cotton worth 20–30 billion US dollars (Anonymous 1997).

1.1. Origin

Cotton has been cultivated for its fibre for more than 5000 years. Despite an aggressive competition from man-made fibre, today it accounts for about half of the world market for textile fiber (Anthony 1991). The wild species of *Gossypium* occur in arid regions of the tropics and subtropics of Africa, Asia, Australia and America. The only cotton variety with spinnable lint that grows wild is *G. herbaceum* var. *africanum*, and this is probably the ancestor of all linted cottons in both the Old and New world (Beasley 1940). All the species of *Gossypium* have been categorized into different genomic groups on the basis of cytology of the interspecific hybrids (Beasley 1942; Phillips & Strickland 1966; Edwards & Mirza 1979). Fryxell (1965) considered that the original dissemination and diversification of the genus *Gossypium* occurred in the Mesozoic period under mesophytic conditions and that adaptation to more xerophytic environments in which *Gossypium* spp. characteristically occur presently began in the early Tertiary period.

1.2. Distribution

The wild species of *Gossypium* are tropical and subtropical in distribution. Commercial production of cotton now extends from 37°N to 32°S in the New World, and from 47°N in the Ukraine to 30°S in the Old World. The cotton growing areas of India fall within 8° to 32° N latitude and 70° to 80° E longitude (Basu 1990). The northern limit of production in the United

States corresponds with the 77°F isotherm and with an average frost-free growing season of 200 days (Purseglove 1988). Thus cotton is now virtually grown all over the world. The main cotton growing areas in the world, average yield per hectare and production have been given in Table 1.1.

Table 1.1: Some important cotton growing areas / countries in the world (1997).

Region/ country	Area Harvested (1000 ha)	Production (1000 tones) (1000 ha)	Yield (kg/ha)
Argentina	884	325	368
Brazil	660	300	455
China	4560	4300	943
India	**8900**	**2856**	**321**
Mexico	197	208	1056
Pakistan	2893	1598	552
Sudan	188	93	495
Turkey	709	755	1065
Egypt	361	315	873
U.S.A	5376	4132	769
World	33815	19737	584

(Source: F.A.O. Production YearBook 1997.)

1.3. Ecology

Cotton is a sun loving plant and cannot tolerate shade particularly in the seedling stage. The optimum temperature for germination is 90°F. The lower temperature increases the production of vegetative branches and extends the cropping period while the higher temperature increases the number of fruiting branches and reduce the cropping period. Reduced light intensity retards flowering and fruiting and increases boll shedding. The crop does not tolerate very heavy rainfall especially if grown as a rain-fed crop. The ideal average rainfall is considered to be about 40-60 inches. In arid areas, it is grown with irrigation. Cotton can be grown in a variety of soils from light sandy soils to heavy alluvium and Rendzina-type clays. Soil aeration, moisture and temperature are important factors in germination and early plant growth (Purseglove 1988).

1.4. Plant habit

The cultivated cottons are shrubs and sub-shrubs (Fig. 1.1A). The plant height ranges from 0.6 to 2.5 m depending on the species, cultivar and the environment. The plant has an erect main stem and is monopodial in habit. Each node above the cotyledon carries a leaf. Leaves are

arranged spirally with a 3/8 phyllotaxy on both the main axis and its vegetative branches (Anthony 1991).

Fig.1.1

Fig. 1.1

A Cotton plants in the field.

B - C. Mature cotton plants with dehisced cotton bolls.

Branches are of two types viz. vegetative and fruiting. A branch meristem is formed in the axil of each leaf and begins development by the differentiation of a prophyll (a small inconspicuous leaf resembling a stipule), an internode and a true leaf. The leaf axillary meristem is responsible for most branching. Fruiting branches are sympodial and their early development begins like the vegetative branches. Flowering proceeds upwards and outwards at regular intervals. Because of the indeterminate growth habit of the plant, buds, flowers and bolls are present at the same time and at all stages of development (Anthony 1991). Fruit called boll, is spherical or ovoid leathery capsule of about 4-6 cm long. Boll grows to full size in about 25 days after opening of the flower. Seeds develop for a further 25 days before the boll opens. On maturity, boll splits along with carpel edges into several valves and expose the lint (Fig. 1.1B, 1.1C). The seed is pyriform and dark-brown in colour after removal of fuzz (Purseglove 1988).

1.5. Economic Importance

Cotton is a multipurpose crop having many economic uses. Seed is the most important part of the plant. It contains two principal components: (a) The hull (outer covering of the seed) from which cotton fiber (lint) and cotton linters arise and (b) kernel or embryo from which oil and meal are obtained. In addition, the seed contains minor constituents such as lecithin, sterols, some vitamins of B and E group and minerals (Pandey 1998). Lint is the most important source of fiber being used in the textile industry. It is an outgrowth of the epidermal cell of the seed.

The seed yields a semi-drying edible oil which is used in lard substitutes (shortening), salad, cooking oil and in margarine manufacture. Low grade oil is used in the manufacture of soap, lubricants, sulphonated oils and protective coatings. The oil content of different cultivated species vary from 16-25% of dry seed weight (Lawhon *et al*. 1977). In India, only a small quantity of cottonseed oil (5-10%) is used for manufacturing of soaps, while a larger quantity is utilized for edible purposes, mostly as edible oil and vanaspati. Brominated cottonseed oils are used for production of flavoured soft drinks. Emulsified cottonseed oil can be used for intravenous administration to patients who require a higher calorie diet (Pandey 1998).

Seed meal or cake, primarily used as a fertilizer and cattle feed contains a high percentage of protein (16-22%) and is rich in essential amino acids like lycine, methionine,

tryptophan and some other amino acids (Beradi & Cherry 1980). Although the meal contains high quality protein, its use as animal feed is restricted to ruminants because of the presence of gossypol (Murray *et al.* 1993). Gossypol is a toxic yellow polyphenolic pigment located in the glands present in embryo, leaves and flower buds etc. Seed may contain as much as 10% gossypol (Fisher *et al*.1988). Gossypol is important as a deterrent to insect pests in cotton (Lukefahr & Houghtaling 1969) and for its pharmaceutical effects in various drugs. During the past few years, gossypol has attracted much attention especially due to its antifertility (Hong *et al.* 1989), antiparasitic (Eid *et al*. 1988), antitumor (Jaroszewski *et al*. 1990; Gilbert *et al*. 1995) and anti-HIV properties (Royer *et al*. 1995). Seed hulls are used for cattle feed and as a soil covering called mulch (Bajaj 1998). It has a poor nutrient value and is generally mixed with cottonseed meal as roughage to increase the volume of the cattle feed. Seed hulls are also used in production of industrially important chemicals such as furfural and active carbon (Pandey 1998).

Linters, which are short fibers hanging to the seed after ginning, are removed at the oil mill. It is a valuable source of cellulose and synthetic fibers etc. (Bajaj 1998). It is used in felts, upholstery, mattresses, twine, wicks, carpets, surgical cotton and in chemical industries such as rayon, plastics, lacquers, paper, photographic films, cellulose explosives and sausage skins etc (Purseglove 1988).

1.6. Current status of cotton in India

India is one of the major cotton producing countries in the world. Cotton is cultivated on 9.1 million hectare in three agroclimatic zones (Northern, central and southern zones). The crop provides the means of livelihood to an estimated 60 million people in India (Basu 1990). Though area under cotton has not appreciably changed since 1970, there is an increase in total production of cotton due to introduction of high yielding varieties and hybrids, use of higher doses of insecticides, proper management of diseases and adoption of improved agricultural practices (Basu 1995). Data on state-wise cotton production, area under cultivation and average yield/ha have been given in Table 1.2.

Table 1.2: State wise cotton production in India (1996 -1997).

States	Area under cultivation (1000 hectares)	Production (1000 bales of 170 kg each)	Average yield (kg / ha)
Andhra Pradesh	1007.4	1848.8	312
Assam	1.7	0.8	80
Bihar	0.0	0.0	0.0
Gujarat	1484.0	2657.0	304
Haryana	649.0	1504.0	394
Himachal Pradesh	0.3	0.3	170
Jammu & Kashmir	0.1	0.2	340
Karnataka	668.1	932.0	237
Kerala	12.3	20.2	279
Madhya Pradesh	526.6	437.2	141
Maharashtra	3084.7	3143.3	173
Manipur	0.1	0.3	510
Meghalaya	7.5	5.3	120
Mizoram	0.1	2.6	442
Orissa	14.0	28.0	340
Pondicherry	0.6	1.0	283
Punjab	742.0	1925.0	441
Rajasthan	654.2	1363.8	354
Tamilnadu	259.5	373.4	245
Tripura	1.1	1.6	247
Uttar pradesh	7.8	7.3	159
West Bengal	0.1	0.3	510
Nagaland	0.2	0.4	340
TOTAL INDIA	**9122.3**	**14252.3**	**279.17**

(Source: Indian Cotton Journal, No: 78, 1997-1998)

India grows stable varieties of all four cultivated species of cotton i.e. *Gossypium hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L. and also F1 hybrids of *intra-hirsutum*, *hirsutum* x *barbadense* and *herbaceum* x *arboreum* (Basu 1995). Out of the total cotton area in the country, F1 hybrids cover 36% area whereas varieties of *hirsutum* are grown on 35.5%, *barbadense* on 0.01%, *arboreum* on 17.0% and *herbaceum* on 11.5 %

area in the country. On the basis of the fibre length, 50% of the total lint production belongs to long (24.5mm – 26mm) and extra-long staple (27mm and above), 45 % to the lower medium (20mm – 21.5mm) + superior medium (22mm – 24mm) and 5 % to the short staple (19mm and below) categories. Hybrids contribute almost 45 % of the total lint production (Basu 1995). A National Gene Bank of cotton genetic resources located at Central Institute for Cotton Research (C.I.C.R), Nagpur, India holds nearly 9000 accessions of all four cultivated species, 25 wild species and a large number of perennial cottons (Basu 1995). Seven important cultivars have been chosen for the present study and details of these cultivars have been given in Table 1.3. Figures on India's import and export of cotton have been given in Table 1.4.

Table 1.3: Details of Indian cultivars of cotton (*G.hirsutum* L) used in the present study.

Characters/ Source etc.	**Cultivars**			
	NHH-44	**DCH-32**	**DHY-286**	**H-8**
Type of hybrid	Intra hirsutum	Inter specific	Intra hirsutum	Intra hirsutum
Place where developed	MAU, Nanded, MS, India	UAS, Dharwad, KN, India	Dr.PDKV, Akola, MS, India	GAU, Surat, GJ, India
Year of release	1985	1983	1978	1983
Area grown	AP, MS, India	KN, A.P, TN, GJ, India	MS, India	GJ, India
Plant habit				
Growth	Annual, Erect	Annual, Erect	Annual, Erect	Annual, Erect
Height	150 cm	150 cm	120 cm	150 cm
Boll size	Roundish medium	Large roundish	Medium	Large
No. of locules	4	3-4	4	4
Crop duration (no. of days)	165 days	180 days	190 days	170 days
Fiber length	25 mm	33 mm	27 mm	30 mm
Staple class	Medium staple	Extra long staple	Medium staple	Long staple
Resistance	Jassid Aphid	-	Jassid	Bacterial blight
Yield (q/ ha)				
Irrigated →	30 – 35 q/ha	35 - 40 q/ha	-	30 - 35 q/ha
Rainfed →	8 – 10 q/h	-	10–12 q/ha	

MAU- Marathawada Agriculture University; UAS – University of Agriculture Sciences; DR. PDKV- Dr. Punjabrao Deshmukh Krishi Vidyapeeth; GAU - Gujarat Agriculture University; MS- Maharashtra State, India; KN- Karnataka State, India; AP- Andhra Pradesh State, India; TN- Tamilnadu State, India; GJ- Gujarat State, India; - Data not available.

Continued -

Characters/ Source etc.	Cultivars		
	CNH – 36	**Anjali LRK-516**	**LRA-5166**
Variety	*G.hirsutum* L.	*G.hirsutum* L.	*G.hirsutum* L.
Place where developed	CICR, Nagpur MS, India	CICR, RS, Coimbatore, India	CICR, RS, Coimbatore, India
Year of release	1993	1992	1982
Area/s where grown	MS, India	MS & TN, India	MS & TN, India
Plant habit			
Growth Boll size	Annual, erect Medium	Annual, erect Big	Annual, erect Medium
No. of locules per boll	4	4-5	4
Crop duration (no. of days)	140	160	165
Fiber length	23 mm	25 mm	26 mm
Staple class	Medium	Superior, Medium	Superior, Medium
Resistance if any	-	Jassid	Drought Tolerant
Yield / ha Irrigated → Rainfed →	 - 8-10 q/ha	 25-30 q/ha 10-12 q/ha	 30-35 q/ha 10-15 q/ha

Source: Personal communication, Regional cotton research station, Sirsa, Coimbatore, India. CICR – Central Institute for Cotton Research; Dr. PDKV- Dr. PunjabRao Deshmukh Krishi Vidyapeeth; RS- Regional station; MS- Maharashtra state, India; TN- Tamilnadu state, India; - Data not available.

Table 1.4: India's Import and Export of cotton.

	Import		**Export**	
Year ending 31st August	**Quantity (Lac bales of 170 Kg each)**	**Value (Rs. in Crores)**	**Quantity (Lac bales of 170 Kg each)**	**Value (Rs. in Crores)**
1996-97	0.30	59.00	16.82	1654.99
1997-98*	3.50	N.A.	3.49	316.85

N.A : Not available, * - year ending 30th September, 1997; 1 Crore = 10 million
Source: Ministry of textiles, Mumbai.

1.6.1. Desi cottons

India is considered to be the birth place of two diploid cotton species *G. arboreum* L. and *G. herbaceum* L. together known as "Desi" cottons ("Desi" literally means native). These two species are highly resistant to pests and diseases, tolerant to drought and are fit for rainfed cultivation in low rainfall and poor soil areas in the country. These species also possess high structural uniformity of fibre with suitability for open end spinning. Inspite of these merits, desi cottons have disadvantages like low yields and short fibre length. Due to these shortcomings, after 1956, desi cottons in India by and large have been replaced by *hirsutum*, an American cotton which is high yielding, provides long and extra long fibre length but is highly susceptible to insect pests. Based on *herbaceum X arboreum* combinations, several desi cotton varieties have been released in the country. At present, 28% of cotton area in country is occupied by desi cottons. Some of the important desi cotton varieties grown commercially in different states of India are AK-5, B-797, Digvijay, G-46, G-22, LD 230, Maljari, Sanjay, Sujay, Suyodhar, Wagad, Western I and Y-1 etc. (Pandey 1998).

1.6.2. Colored cotton

Naturally pigmented cottons (colored cottons) are a new arrival on the western fashion market. Textiles made from colored cottons are eco-friendly and do not require artificial dyes. Because of its rarity, these cottons fetch much higher price compared to white ones. Very little is known about the history of colored cottons. These have been reported to be occurring even 5,000 years ago. Fossils obtained in northern coastal Peru have shown the existence of blue, purple, pink, green, brown and red colored cottons. Presently, colored cottons (black, green and brown) are mostly grown in the American continent on a very limited scale. A research group in Peru led by Dr.Vreeland is actively engaged in producing cotton clothes with colored cottons and selling them internationally under the brand name "Pakacho" which means brown cotton. Dr. Vreeland first discovered the naturally occurring colored cotton in Peru in 1977. Some 15.000 peasants and Indians who grow these cottons in dozens of plots throughout Peru are by far the largest group of producers of colored cottons in the world (Vreeland 1999).

Except for the pigmentation of the fibre, the color cottons physically resemble normal white cotton. Short staple length, weak fibre strength and low micronaire value are some of the characteristics of colored cottons. These have properties of insect, disease and drought resistance. The major disadvantage of the colored cottons (Fig. 1.2A) is the transfer of colored

trait to white varieties by cross pollination resulting contamination of the white varieties and lower market value of the lint. This problem could be overcome by cultivation of colored cottons under strict legislation and in isolated areas (Anonymous 1998).

In India, colored cottons "Red Northerns" and "Coconadas" were grown and exported to Japan and some European countries until 1960. Brown cotton was grown in Tripura and Andhra Pradesh's Kakinada areas until the 80s. Recently, a research group at Agricultural Research Station, Dharwad, Karnataka, India has developed a stable variety of almond colored cotton known as "Dharwad Deshi Colour Cotton–1" (DDCC-1). The variety is under evaluation for release (Anonymous 1998).

1.7. Factors affecting cotton production

1.7.1. Abiotic Stresses

Abiotic stresses such as cold, drought and heat have negative effects on the cotton yield (John 1997). Under water stress, cotton plants reduce root and shoot growth differentially, increasing the root/ shoot ratio (Malik *et al*. 1979; Ball *et al.* 1994). Salinity induces nutritional imbalances (Martinez & Lauchli 1994) and affects the cotton growth, yield (Nawar *et al.* 1994) and fiber quality (Razzouk & Whittington 1991).

1.7.2. Biotic stresses

1.7.2.1. Insect pests

Various insect pests and diseases cause a tremendous loss in cotton production. The cost of damage caused by insects alone has been estimated to be over 600 million US $ per year (Perlak *et al*. 1990) and over 200 million US$ are spent annually to crop protection from

insects (Jenkins *et al.* 1991b). Cotton growers use almost half of the

Fig. 1.2

A. Naturally pigmented brown cotton.

insecticides applied to crops in the United States (Adkisson *et al.* 1982). About 100 species of insects are known to be associated with cotton (Berger 1969). The most serious pests of cotton are bollworms: *Heliothis* zea Boddie and *H. armigera* Hubn. are distributed in America and Africa respectively. These bollworms are the caterpillars of several species of moths. The caterpillar feed in the boll (Fig.1.3A) damaging lint and seeds and cause a considerable reduction in yield and quality. Three fourth of the damage to cotton in the United States is caused by the boll weevil and the cotton bollworms (Pendergrass 1989). Boll weevil (*Anthonomus grandis* Boh.) is the worst pest in the United States, which attacks the young squares, bolls and terminal buds. The other major insect pests are pink bollworm (*Platyedra gossypiella* Saund.) which are widely distributed in Africa, Asia and America and spiny bollworm (*Earias biplaga* Wlk.) and *E.insulana* Boisd. found in Africa and Asia. Leaf, stem and bud sucking bugs also cause considerable damage to cotton (Purseglove 1988).

In India, the major insect pests causing considerable economic loss in cotton production are American bollworm, pink bollworm, spotted bollworm and different sucking pests like jassids, aphids and whitefly. Beside these, leaf eating caterpillars (*Spodoptera litura*) and stem weevil also damages the crop in some areas in south of India. Aphids, jassids and thrips damage the crop in early stages of plant development while different bollworms and whitefly generally infest the crop during reproductive phase of the crop (Sundaramurthy *et al.* 1990). The first outbreak of American bollworm (*H. armigera* Hubn.) was observed in 1987-88 in Andhra Pradesh, while the outbreak of Whitefly was observed during 1984-1985 in Andhra Pradesh and some parts of Karnataka, Tamilnadu and Maharashtra. The average cotton production in Andhra Pradesh dropped from 6.08 q/ha in 1983/84 to 2.63 q/ha in 1987-88 by American bollworm and whitefly. In severe cases, losses upto 75% in some areas have been accounted due to *Heliothis.* Monocropping, indiscriminate use of insecticides, drought, insect's resistance to insecticides, availability of other susceptible crops, excessive use of nitrogenous fertilizers are some of the important factors responsible for *Heliothis* outbreak in India. During 1997, sudden outbreak of leaf caterpillar (*Spodoptera*) in Andhra Pradesh reduced the yields from 15 q/ha to 3 q/ha. This resulted in many suicides by farmers who have failed to return the loans taken from moneylenders for purchase of pesticides to save their crop (Bharathan 2000). In nutshell, insect menace still remains a serious concern in India, in spite of the fact that out of total chemical

pesticides used in crop protection in the country, almost 50 % of them are required for cotton crop alone (Joshi 1995). A list of major insect pests causing damage to cotton crop is given in Table 1.5.

Table 1.5: Insect pests of cotton crop.

No.	Insect pests	Scientific names
1.	Boll weevil	*Anthonomous grandis*
2.	Spotted bollworm	*Earis spp.*
3.	American bollworm	*Heliothis spp.*
4.	Pink boll worm	*Pectinophora gossypiella*
5.	Leaf worm	*Alabama argillacea*
6.	Aphid	*Aphis gossypii*
7.	Thrips	*Thrips tabaci*
8.	Flea hopper	*Psallus seriatus*
9.	Tarnished and rapid plant bugs	*Lygus hesperus*
10.	Jassid	*Amrasca biguttuals biguttula*
11.	Whitefly	*Bemisia tabaci*
12.	Cabbage looper	*Trichoplusiani*

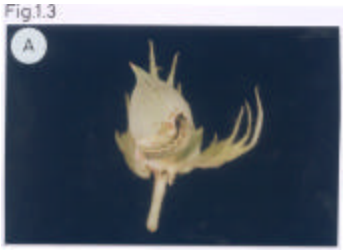


Fig. 1.3

A. Damage caused to cotton boll by the insect *Heliothis*, commonly known as bollworm.

1.7.2.2. Major diseases

Cotton is also affected by some of the major bacterial and viral diseases. The most common diseases are Bacterial blight, Leaf spots, Grew mildew, Wilts and Root rot. Some of the diseases are widely spread throughout the cotton growing areas in India, while other diseases are location specific (Basu 1995).

The disease, bacterial blight caused by *Xanthomonas malvacearum* (E. F. Sm.) Dowson. has now spread to most cotton growing countries in the world. Infection of this disease

is carried out by seed and plant debris. The symptoms of the disease are water soaked lesions on the cotyledons, leaves and on the bolls. The infection on the boll later produces blackened lesions which ultimately result premature opening and shedding of the bolls.

Fusarium wilt, caused by *Fusarium oxysporum* Schlecht. *F. vasinfectum* (Atk.) Synder & Hansen, is a soil-borne fungal disease. This disease particularly affects diploid cottons (Basu 1995). It causes death or stunting of the plant with yellowing and wilting of leaves and discoloration of the woody portion of the stem.

Verticillium wilt caused by *Verticillium alboatrum* Reinke & Berth. is another soil borne disease. The disease is aggravated by cold wet weather and irrigation. Stunting, chlorotic, mottling and shedding of the leaves, squares and bolls are the symptoms of this disease.

Cotton is also affected by leaf curl virus. The disease is transmitted by the white fly, *Bemisia tabaci* (Genn.) (syn. *B. gossypiperda* M.& L.). In the affected plants, all parts of the stem become twisted and spindly, leaves curl and crinkle, veins thicken and chlorotic spots and streaks develop in the lamina (Purseglove 1988). In India, the disease has now spread to Haryana, Punjab and Rajasthan states and may become a potential threat to cotton cultivation in the country (Basu 1995).

Leaf spots disease caused by *Alternaria and Myrothecium* create havoc under favorable climatic conditions. *Alternaria* leaf spot is very severe on diploid cottons in some parts of Karnataka (India) while *Myrothecium* leaf spot has been found to occur in Haryana (India).

Grew mildew which has been very severe on diploid cottons has now started affecting tetraploid cottons (Basu 1995). Nematodes also cause considerable losses in cotton yield and quality. In 1992, in the USA alone, they accounted for yield loss of 528000 bales valued at millions of US$ (Goodell 1993). Names of diseases and their causal organisms affecting cotton crop have been given in Table 1.6.

Table 1.6: Some common diseases affecting cotton.

Type of Causal organism	No.	Disease	Name of the causal organism
Bacteria	1	Bacterial blight	*Xanthomonas malvacearum*
Fungus	1	Ascochyt blight	*Ascochyta gossypii*
	2	Anthracnose	*Glomerella gossypii*
	3	Fusarium wilt	*Fusarium oxysporum f. vasinfectum*
	4	Cotton rust	*Puccinia cabata*
	5	Verticillium wilt	*Verticillium alboatrum*
Nematode	1	Root rot	*Phymatotrichum omnivorum*
	2	Root knot nematode	*Meloidogyne incognita*
	3	Rhizoctonia root rot	*Rhizoctonia solani*
Virus	1	Leaf curl Virus	*Bemisia tabaci* (Vector)

1.8. Improvement of cotton

The main objectives of cotton improvement include increase in yield, fibre quality, early maturity, gossypol free seed and development of resistance to various insects, diseases and nematodes (Bajaj 1998).

1.8.1. Conventional methods

Cotton is a self-pollinated plant, but, depending on the presence of suitable insects, cross pollination can occur. Hybridization and mutation breeding have often been used to introduce variability into populations. India has distinction in the world to commercially exploit the phenomenon of heterosis in cotton by conventional plant breeding methods. The production of cotton in the country has improved drastically by growing a large number of F1 hybrids since early 70's. Due to easy availability of cheap and skilled farm workers, a number of hybrids have been developed by hand emasculation and pollination (Basu 1990). Currently a large number of F1 hybrids are grown all over India covering more than 36% of the total cotton area i.e. 9.1 million hectares and contributing more than 45% of the total lint production in the country (Basu 1995).

1.8.2. Need for Non conventional methods

Production of hybrid cottons in India has lead to a spectacular improvement in yield and fibre traits. However, this approach has not been followed in other countries as the process is laborious and time consuming and due to high costs involved in hand pollination (Srinivasan *et al.* 1972; Davis 1978).

Wild species of *Gossypium* although are short fibered or lintless but possess a number of useful traits which are mentioned in Table 1.7. Transfer of some of these traits from the Old World diploids to the cultivated tetraploids has been unsuccessful over the years due to incompatibility barriers and abortion of hybrid embryos (Weaver 1958; Pundir 1972). Hence, there is a strong need to resort to non-conventional biotechnological methods for improvement of cotton species.

Table 1.7: Wild species of cotton possessing useful traits.

No.	Species	Useful traits
1.	*G. somalense* (Gurke) J.B.Hutch.	Resistance to bollworm
2.	*G. armourianum* Kearn.	Resistance to bollworm, increased number of ovules per loculus
3.	*G. thurberi* Tod.	Resistance to bollworms, high fiber strength
4.	*G. raimondii* Ulbr.	Resistance to jassids and tolerant to drought, high density of seed hairs
5.	*G. harkensii* Brendeg.	Resistance to drought and spider mites, source of cytoplasmic male sterility
6.	*G. anomalum* Wawrex Wawra & Peyr	Lint quality, resistance to jassid
7.	*G. aridum* (Rose & Standl.) Skov.	Tolerance to drought
8.	*G. stocksii* Mast.in Hook.	Resistance to drought
9.	*G. tomentosum* Nutt.ex Seem.	Resistance to drought and jassids, lint quality
10.	*G. bickii* Prokh.	Gossypol – free seeds

(Source: Bajaj 1998)

1.9. Biotechnological approaches

During the last two decades, a number of reports have been published on various aspects of biotechnological studies on cotton such as callus initiation, somatic embryogenesis, organogenesis, protoplast culture, interspecific hybridization through embryo or ovule culture, somaclonal variation and plant transformation etc. These reports have been summarized in the following sections.

1.9.1. Callus initiation

There are several reports on establishment of callus cultures in cotton (Table 1.8). Induction of callus had been achieved from almost every part of the cotton plant like hypocotyl, mesocotyl, cotyledon, root, leaf, petiole, stem, embryo, anther, ovule and protoplast etc. The main objectives of callus initiation in these reports have been to conduct studies for isolation and culture of protoplasts, production of somatic hybrids, establishment of suspension culture for organogenesis. However, differentiation of callus in all these reports could not be achieved.

Table 1.8: Studies on *In vitro* callus initiation.

No.	Species	Explant used	Reference
1.	*G. hirsutum* L.	MC	Schenk & Hilderbrandt (1972)
2.	*G. hirsutum* L.	L	Davis *et al.* (1974)
3.	*G. hirsutum*,L.;*G. arboreum* L.	H & S	Rani & Bhojwani (1976)
4.	*G. barbadense* L. *G. davidsonii* Kell.	COT	Katterman *et al.* (1977)
5.	*G. anomalum* Wawr. Ex Wawr. & Peyr.; *G. arboreum* L.; *G. armourianum* Kearn.; *G. hirsutum*,L.; *G. klotzschianum* Anderss *G. raimondii* Ulbr.	H	Price *et al.* (1977)
6.	*G. arboreum* L. *G. hirsutum* L.	H	Smith *et al.* (1977)
7.	*G. hirsutum*, L. *G. arboreum* L.	H	Bajaj & Gill (1985)
8.	*G. arboreum* L. *G. hirsutum* L.	H	Zimmerman & Robacker (1988)
9.	*G. arboreum* L.	ANT	Bajaj & Gill (1989)

ANT - Anther; H - Hypocotyl; MC - Mesocotyl; COT - Cotyledon; L - Leaf; S - Stem.

1.9.2. Somatic embryogenesis

The first report on induction of somatic embryogenesis in a wild species of cotton was published by Price & Smith (1979), however, somatic embryos could not develop into plantlets. The first successful regeneration of whole cotton plant *via* somatic embryogenesis was obtained by Davidonis & Hamilton (1983), however, the method had limitation due to long incubation period of callus for induction of proembryoids and low efficiency of embryo formation. In a different study, Shoemaker *et al*. (1986) evaluated seventeen *G. hirsutum* L. cultivars for induction of somatic embryogenesis and plant regeneration. Approximately 40% of the somatic embryos underwent normal germination and the procedure was simple and rapid. Somatic embryogenesis from callus cultures of mature leaf and petiole explants from six cotton varieties has been reported by Gawel *et al*. (1986). Trolinder & Goodin (1987, 1988 a, b) could achieve the regeneration of cotton plants from embryogenic suspension cultures. They concluded that induction of somatic embryogenesis in cotton is genotype dependent. Finer (1988) also reported plant regeneration from somatic embryogenic suspension cultures established from cotyledons of cultivar Coker 310. Plant regeneration in Indian cultivar of cotton MCU-5 through somatic embryogenesis was first reported by Kumar & Pental (1998 a, b). Cotton cultivar MCU 5 and few others (Khandwa 2, Bikeneri Norma, F 846, MCU 7 and barba 11-98) were crossed with fully regenerating lines of Coker 310. The resulting F1 hybrids showed regeneration (20-59% of explant) *via* somatic embryogenesis. MCU 5 produced highest number of somatic embryo (14.28 per explant).

In the reports published so far, it has been observed that *in vitro* regeneration by somatic embryogenesis in cotton is a genotype dependent phenomenon. A few Coker varieties have been reported to have the highest regeneration potential compared to other varieties. However, plants regenerated *via* somatic embryogenesis have shown phenotypic changes (Stelly *et al.* 1989). Studies on somatic embryogenesis carried out so far in cotton have been listed in Table 1.9.

Table 1.9: Studies on somatic embryogenesis in cotton.

No.	Genotype	Explant Used	Mode of Regeneration	Reference
1.	*G.klotzschianum Anderss*	H	C∏SE	Price & Smith (1979)
2.	*G.hirsutum* L.	COT	C∏PE ∏PT	Davidonis & Hamilton (1983)
3.	*G.klotzschianum* Anderss.	ST, P, LD	C∏SE	Finer & Smith (1984)
4.	*G.hirsutum* L.	H, IE	C∏SE∏PT	Rangan *et al.* (1984)
5.	*G.hirsutum* L.	H	C∏SE∏PT	Shoemaker *et al.* (1986)
6.	*G.hirsutum* L	LD. P	C∏SE∏PT	Gawel *et al.* (1986)
7.	*G.hirsutum* L.	H	C∏SE∏PT	Umbeck *et al.* (1987)
8.	*G.klotzschianum Anderss*	H	C∏SC∏SE∏PT	Finer *et al.* (1987)
9.	*G.hirsutum* L.	H	C∏SE∏PT	Tolinder & Goodin (1987)
10.	*G.hirsutum* L.	H	C∏SE∏PT	Tolinder & Goodin (1988 a)
11.	*G.hirsutum* L.	H	C∏SE∏PT	Tolinder & Goodin (1988 b)
12.	*G.hirsutum* L.	COT	C∏SC∏SE∏PT	Finer (1988)
13.	*G.hirsutum* L. *G.barbadense* L. *G.arboreum* L.	H	C∏SE	Trolinder & Xhixian (1989)
14.	*G.hirsutum* L.	P	C∏SE	Gawel & Robacker (1990 a)
15.	*G.hirsutum* L.	H	C∏SE∏PT	Voo *et al.* (1991)
16.	*G.hirsutum* L.	COT, H	C∏SE∏PT	Firoozabady & De Boer (1993)
17.	*G.hirsutum* L.	H	C∏SE∏PT	Kumar & Pental (1998 a)
18.	*G.hirsutum* L.	H	C∏SE∏PT	Kumar & Pental (1998 b)
19.	*G.hirsutum* L.	COT, H	C ∏ SE∏PT	Zhang *et al.*(2000 a)

C – Callus; SC - Suspension Culture; SE - Somatic Embryo; P - Petiole; PT - Plantlet; H- Hypocotyl; PE – Pro-Embryo; LD - Leaf Disc; IE - Immature Embryo; COT – Cotyledon; ST – Stem.

1.9.3. Organogenesis

Regeneration of plants *via* pre-existing meristems has been used as an alternative approach for development of true to type plants, independent of genotypes. *In vitro* culture of excised meristems of *G. hirsutum* L. was first reported by Chappel & Mauney (1967).

Although new leaf primordia could be initiated, the root system failed to develop. Bajaj & Gill (1986) reported plant regeneration from shoot tips of the field grown plants of *G. hirsutum* L. and *G. arboreum* L. Induction of multiple shoots and plant regeneration from decapitated cotyledonary nodes in cotton was first reported by Agrawal *et al*. (1997). These and many other reports on plant regeneration from pre-existing meristems in cotton have been listed in Table 1.10.

Table 1.10: Studies on plant regeneration in cotton *via* pre-existing meristems.

No.	Species	Explant used	Response	Reference
1.	*G.arboreum* L. *G hirsutum* L.	M, ST	Adventitious buds & multiple shoots	Bajaj & Gill (1986)
2.	*G.hirsutum* L. *G.barbadense* L.	SA	Single shoots	Gould *et al.* (1991)
3	*G hirsutum* L.	CN – SA	Multiple shoots	Agrawal *et al.* (1997)
4.	*G.hirsutum* L. *G.arboreum* L.	SA + 2 C, SA + 1C, SA - 2C	Multiple shoots	Gupta *et al.* (1997)
5	*G.hirsutum* L.	ST	Single shoots	Sayeed *et al.* (1997)
6.	*G hirsutum* L.	SEA	Single shoots	Agrawal *et al.* (1998)
7. 8.	*G.hirsutum* L. *G.hirsutum* L.	PM CA	Multiple shoots Multiple shoot	Hemphill *et al.* (1998) Morre *et al.* (1998)
9	*G. hirsutum* L.	SA	Single shoots	Zapata *et al.* (1999 b)
10.	*G. hirsutum* L *G.arboreum* L.	CN, SCN, ST, PB	Multiple shoots	Hazra *et al.* (2000)

M- Meristem; ST – Shoot Tip, SA- Shoot Apex; 2C- Two Cotyledon; 1C- One cotyledon SEA- Split Embryo Axis; PM – Preexisting Meristem; CN- Cotyledonary Node; CA- Caulinar Apex; SCN- Split Cotyledonary Node; PB- Petiole Base.

1.9.4. Embryo Rescue

Inter-specific hybrid production through embryo rescue technique has been achieved by many workers. Success with various wild and cultivated cotton species, both diploid and tetraploid achieved by Bajaj and Gill has been reviewed (Bajaj & Gill 1998). The hybrids were obtained by preventing the degeneration of embryos by treating the flowers / young bolls with growth regulators and then culturing the rescued embryos / ovules on defined nutrient media. Establishment of crosses between diploid and tetraploid species like *G. arboreum* L. X *G.*

hirsutum L. (Gill & Bajaj 1987), *G.trilobum* (Moc. & Sess.ex DC.) Skov.emend.Kearn. X *G. hirsutum* L. (Umbeck & Stewart 1985) and *G.sturtianum* J.H.Willis X *G. hirsutum* L. (Altman *et al.* 1987) have also been reported.

1.9.5. *In vitro* fertilization

Hybrid production in incompatible crosses has been successfully achieved by *in vitro* pollination and fertilization technique. This involves culturing of unpollinated flowers, ovaries and ovules on synthetic media and then sprinkling of pollen over them or on the cut end of the style. Rafaat *et al*. (1984) obtained hybrids by fertilizing *G.hirsutum* L. *in vitro* with pollen of *G.barbadense* L. Later in a separate study, Liu *et al*. (1992) succeeded in producing hybrids between *G.hirsutum* L. and *G.arboreum* L. by this technique. Although *in vitro* fertilization resulted in the formation of seeds, however their frequency was rather low. Factors like media, temperature and relative humidity plays a crucial role in the fertilization rate.

1.9.6. Ovule Culture

Cotton ovule culture has provided a valuable tool to circumvent problems encountered in hybridization of diploid and tetraploid species. In addition, ovule culture has also been applied for understanding the development of cotton fibres *in vitro*. The phytohormone regime required for successful development of ovule to whole plant seems to be critical and may vary with each parental germplasm set. Thus, the full potential of ovule culture for improvement of commercial cotton germplasm remains to be tapped. Reviews on cotton ovule culture describing methods, applications and successful reports have been published (Stewart 1991; Beasley 1992).

1.9.7. Protoplast culture

Protoplast as an explant has been used for direct gene transfer because of the freely accessible plasmalemma and non-involvement of biological vector in the transformation process (Peeters *et al*. 1994). Though protoplasts isolated from cotton cotyledons (Khasonov & Butenko 1979), hypocotyl callus (Bhojwani *et al*. 1977; Finer & Smith 1982; Firoozabady & Deboer 1986) and stem callus (Saka *et al*. 1987) could not be regenerated, successful plant regeneration from protoplasts derived from embryogenic cell suspensions has been reported (Chen *et al*. 1989; She *et al*. 1993; Peeters *et al*. 1994). Reports on protoplast culture in cotton have been listed in Table 1.11.

Table 1.11: Studies on protoplasts in cotton.

No.	Species	Donor tissue	Response	Reference
1.	*G.hirsutum* L.	HC	Macro colonies	Bhojwani *et al.* (1977)
2.	*G.klotzschianum* Anderss.	HC	Macro colonies	Finer & Smith (1982)
3.	*G.hirsutum* L.	AC	Callus	Thomas & Katterman (1984)
4.	*G.hirsutumi* L. *G.barbadense* L.	COT	Micro colonies	Firoozabady & DeBoer (1986)
5.	*G.hirsutum* L.	SC	Callus	Saka *et al.* (1987)
6.	*G.hirsutum* L.	ES	Plant, Micro colonies	Chen *et al.* (1989) She *et al.* (1993)
7.	*G.hirsutum* L.	ES	Fertile plant	Peeters *et al.* (1994)

HC - Hypotcotyl Callus; COT - Cotyledon; SC - Stem Callus; AC - Anther Callus; ES - Embryogenic Suspension.

1.9.8. Somaclonal Variations

The Plant cell cultures on prolonged storage at normal temperature or on periodical subculturing, undergo genetic aberrations such as endomitosis, chromosome loss, polyploidy, translocations, gene amplifications and mutations etc. (D'Amato 1985; Bajaj 1990). These changes are collectively referred to as "somaclonal variations" are a rich source of genetic variability. Bajaj & Gill (1985) have reported chromosomal changes in *in vitro* cell cultures of cotton. Stelly *et al*. (1989) observed that somaclonal plants regenerated from callus cultures of *G.hirsutum* L. extremely varied in phenotypic characters. In another study, Trolinder & Xaiomin (1991) developed high temperature resistant cotton plants from selected somaclones of *G. hirsutum.*

1.9.9. Genetic transformation of cotton *via Agrobacterium tumefaciens* (AT)

Genetic transformation in cotton *via* A*grobacterium tumefaciens* mediated technique was first reported by Firoozabady *et al*. (1987) and Umbeck *et al*. (1987). However, the first transgenic cotton expressing *Bacillus thuringiensis* var. kurstaki (*cryIA (b)* and *cryIA(c)* genes

and conferring resistance to insects was reported by Perlak *et al*. (1990). Bayely *et al*. (1992) and Lyon *et al*. (1993) have engineered the 2,4-D resistance trait in cotton by transferring the 2,4-D mono oxygenase gene *tdf*A from *Alcaligenes eutrophus*. Herbicide resistant transgenic cotton carrying mutant forms of a native acetohydroxyacid synthase (AHAS) gene have been obtained (Rajasekaran *et al*. 1996 b). The expression of Protease inhibitor gene in cotton plant has also been reported (Thomas *et al*. 1995). In a recent study, Zapata *et al.* (1999 a) used shoot apex as an explant for *Agrobacterium* mediated transformation in cotton compared to earlier reports of regeneration of transformants through somatic embryogenesis.

1.9.10. Genetic transformation by Particle bombardment method (PB)

Finer & McMullen (1990) and Rajasekaran *et al.* (2000) bombarded embryogenic cell suspensions with chimeric genes and developed transgenic plants via somatic embryogenesis. In other cases, meristems of embryo axis explants derived from seeds were used as targets for bombardment to develop transgenics in cotton (McCabe & Martinell 1993; Chlan *et al.* 1995; Keller *et al.* 1997). Reports on cotton transformation *via Agrobacterium tumefaciens* and particle bombardment mediated techniques have been listed in Table 1.12.

1.9.11. Genetic engineering of cotton fiber

Cotton is the premier natural fiber for textile industry. Over the last several decades, significant improvement has been made in the physical properties of cotton fiber through classical plant breeding. However, to make cotton fiber more versatile for textiles, there is a need to improve not only its strength and length but also its dye binding, thermal, wrinkle and shrinkage resistance properties. Recombinant DNA technology and improved transformation methods may enable production of new and improved fibers that could compete with synthetic ones (John 1994, 1996). Cotton has been transformed for fibers having better insulating characteristics. To achieve this, engineered *phaB* (acetoacetyl-CoA reductase) and *phaC* (polyhydroxyalkanoate synthase) genes from *Alcaligenes eutrophus* were used in a particle bombardment mediated transformation. As a consequence, the rate of heat uptake and cooling was slower in transgenic fibers, resulting in higher heat capacity (John 1996). In another report, production of thermoplastic polymer polyhydroxybutyrate (PHB) in cotton fibers was obtained through particle bombardment mediated plant transformation (Rinehart 1996).

Table 1.12: Studies on genetic Transformation in cotton by *Agrobacterium* and particle bombardment methods.

No.	Species	Explant Used	Method Used	Response	Reference
1.	*G. hirsutum* L.	COT	AT	SE	Firoozabady *et al.* (1987)
2.	*G. hirsutum* L.	H	AT	SE	Umbeck *et al.* (1987)
3.	*G. hirsutum* L.	ECS	PB	SE	Finer & McMullen (1990)
4.	*G. hirsutum* L.	H	AT	SE	Perlak *et al.* (1990)
5.	*G. hirsutum* L.	H	AT	SE	Bayley *et al.* (1992)
6.	*G. hirsutum* L.	M	PB	SS	McCabe & Martinell (1993)
7.	*G. hirsutum* L.	COT	AT	SE	Thomas *et al.* (1995)
8.	*G. hirsutum* L.	M	PB	SS	Chlan *et al.* (1995)
9.	*G. hirsutum* L.	CS	AT & PB	SE	Rajasekaran *et al.* (1996 b)
10.	*G. hirsutum* L. *G.barbadense* L.	M	PB	SS	Keller *et al.* (1997)
11.	*G. hirsutum* L.	SA	AT	SS	Zapata *et al.* (1999 a)
12.	*G. hirsutum* L.	ECS	PB	SE	Rajasekaran *et al.* (2000)

SE - Somatic embryo; AT - *Agrobacterium tumefaiciens;* PB – Particle bombardment; SS- Single shoot; SA - Shoot apex; ECS - Embryogenic cell suspension. M - Meristem; COT - Cotyledon; H - Hypocotyl.

1.10. Transgenic cotton – present status

1.10.1. Insect resistance Bt (*Bacillus thuringiensis*) cotton

Cotton is one of the few transgenic crops successfully commercialized in the USA. Insecticidal protein genes of *Bacillus thuringiensis* var. *kurstaki* Cry 1A(b) and Cry 1A(c) have been incorporated and expressed into cotton cultivar Coker 310 *via Agrobacterium tumefaciens* mediated transformation (Perlak *et al.*1990, 1991). After several years of field testing, Monsanto's Bollgard cotton containing Bt genes was first released to farmers in the USA in 1996. Bollgard cotton was planted in 13% of the US cotton areas (over 1.8 million

acres) in 1996 which resulted an average yield improvement of 7% (56 pounds per acre yield advantage) compared to non Bollgard varieties (Anonymous 1997). Extensive field testing of transgenic Bt cotton indicated that some of the most damaging insect pests can be controlled (Jenkins *et al.* 1991 a, b; Wilson *et al.* 1994). In 1998, over 100,000 hectares of Bt cotton were planted in China. Transgenic cotton resistance to *Fusarium* and *Verticillum* Wilt diseases has also been developed in China and is expected to reach the market by 2000 or 2001 (Zhang *et al.* 2000 b).

1.10.2. Herbicide resistance

One of the most commonly used herbicide to control broadleaf weeds is 2,4-dichlorophenoxyacetic acid (2,4-D). Cotton has been engineered for resistance to 2,4-D (Bayley *et al*. 1992; Lyon *et al*. 1993). The 2,4-D monooxygenase gene *tdf*A isolated from *Alcaligenes eutrophus* plasmid pJP5, was modified and expressed in cotton plants. The plants obtained were tolerant to 2,4-D, three times the field level of the herbicide used for wheat, corn and sorghum. Herbicide resistant transgenic cotton harboring a single copy of *tdf*A gene is now under field trials (Bayley *et al*. 1992). In another study, Rajasekaran *et al.* (1996 b) transformed Acala and Coker varieties resistant to imidazolinone herbicides. It was observed that transgenic progeny plants were resistant to imidazolinone herbicides at five times the field application level. "BXL" cotton tolerant to bromoxynil and "Roundup Ready"cotton tolerant to glyphosate, a biodegradable herbicide have been developed and marketed in the USA. Surveys on "Roundup Ready"cotton, conducted by Monsanto in September, 1997, showed that approximately 90% of more than 1700 growers expressed satisfaction over the new technology. On an average, they planted 18% of their cotton acreage to "Roundup Ready"cotton in 1997. The advantages enumerated for planting "Roundup Ready" cotton include: weed control, reduced labour needs, reduced input costs and crop safety etc. (Anonymous 1998). In China, cotton varieties resistant to 2.4-D and bromoxynil are expected to be released by 2001-2002 (Zhang *et al.* 2000 b).

Although tremendous progress has been achieved in this area, however, in India, efforts in several Institutes are underway to develop transgenic cotton resistant to insects. An American multinational giant, Monsanto has formed a joint venture with Mahyco Biotech. Pvt. Ltd. (India) to develop insect resistant cotton cultivars and conduct field trials in India. The Government of

India has recently allowed Monsanto to conduct field trials of its genetically engineered cotton on 40 sites located in eight states before it could be released to farmers (Anonymous 1999).

1.11. Aims of the thesis

From the literature survey it becomes obvious that Indian cotton cultivars are recalcitrant. At the time of initiation of the present study, there were no reports of regeneration with Indian cultivars and majority of the work is with American Coker varieties. Transformation studies and transgenic cotton plants generated are with Coker varieties and introduction of such varieties under Indian conditions may not lead to high yields due to lack of adaptability. Therefore any genetic improvement of Indian cultivars of cotton for their use under different climatic conditions in India needs extrapolation of the work already done with Coker varieties to Indian cultivars which is a major pre-requisite.

The objectives of the thesis are therefore aimed at fulfilling these pre-requisites so that Indian cultivars of cotton with agronomically desirable trait through biotechnological methods could be evolved.

1. to develop highly reproducible and efficient *in vitro* plant regeneration methods from explants with pre-existing meristems,
2. the study of the effect of antibiotics on frequency of shoot formation from pre-existing meristems such as embryo axis.
3. to standardize *Agrobacterium* mediated genetic transformation of cotton embryo axes and their molecular characterization.
4. to develop an *in vitro* micrografting as a method for regeneration of putative transformants growing slow and difficult to root.
5. and to optimize different parameters of particle bombardment method for transient gene expression in embryo axis explants as an alternate approach of plant transformation.

CHAPTER 2

MATERIALS AND METHODS (General)

This chapter describes the techniques routinely followed in plant tissue culture work. Techniques of genetic transformation by *Agrobacterium and* Particle bombardment methods used in the present study have been described in the respective chapters (chapter 5 and 6) of the thesis.

2.1. Glassware

Glassware used in all the experiments was procured from "Borosil", India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), petri dishes (85 mm x 15 mm), conical flasks (100, 250, 500 and 1000 ml capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

2.1.1. Preparation of Glassware

Glassware used for all the experiments was cleaned by boiling in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Thereafter, it was immersed in 30% nitric acid solution for 30 min followed by repeated washing in tap water. Washed glassware was thereafter rinsed with distilled water and dried at room temperature (ambient temperature) or in an oven at 200 °C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd, Andhra Pradesh, India). Pipettes and petri dishes were wrapped in brown paper and then sterilized in autoclavable polypropylene bag. Autoclaving of the glassware and above items was done at 121°C, 15 lb psi for 1 h.

2.2. Plasticware

Sterile disposable filter sterilization units and petri dishes (35 mm, 55 mm and 85 mm diameter) were procured from "Laxbro", India. Eppendorf tubes (1.5 ml and 2 ml capacity), microtips (0-200 µl and 200-1000 µl capacity) were also obtained from "Laxbro" and "Tarsons", India. Wide bore microtips (0-200 µl) were procured from "Sigma", USA.

2.3. Chemicals

All chemicals used in the tissue culture study were of analytical grade and were obtained from " Qualigens", "S.D fine chemicals" or "Hi-media", India. The chemicals used in molecular biology study were obtained from "Sigma Chemical Co.," USA. Growth regulators, vitamins, antibiotics (except Cefotaxime) and Phytagel were also obtained from "Sigma Chemical Co.", USA. Cefotaxime was procured from Russel India Ltd. Bombay,

India. Sucrose, glucose, gelling agent and agar-agar were obtained from "Qualigens" and "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO®" laboratories, USA.

2.4. Preparation of culture media

Double distilled water was used for preparation of the media used in the study. After addition of all macro- and micro-nutrients, vitamins, growth regulators and other necessary carbohydrate source like sucrose or glucose, the pH of the media was adjusted to 5.8 before autoclaving using 0.1N NaOH or HCl. Volume was made and gelling agent was added as per requirement. The medium was steamed to melt the gelling agent. Melted medium was then dispensed into test tubes, flasks and thereafter sterilized by autoclaving at 121°C at 15 lb psi for 20 min. Thermolabile growth regulators and antibiotics were filter sterilized through a millipore membrane (0.22μm or 0.45μm pore size). These were added to autoclaved medium before dispensing. Compositions of Murashige and Skoog's (MS) and Gamborg's (B5) macro-, micro elements and vitamins used in the present study are given in Table 2.1, 2.2 and 2.3 respectively.

Table 2.1: Composition of macro-element salts of MS (Murashige & Skoog 1962) and B5 (Gamborg *et al.* 1968) basal media.

Macro-element	MS (mg/l)	B5 (mg/l)
KNO_3	1900	2500
NH_4NO_3	1650	-
$CaCl_2.2H_2O$	440	150
$MgSO_4.7H_2O$	370	250
KH_2PO_4	170	-
$NaH_2PO_4.H_2O$	-	150
$(NH_4)_2\ SO_4$	-	134

Table 2.2: Composition of micro-element salts in MS (Murashige & Skoog 1962) and B5 (Gamborg *et al.* 1968) basal media.

Micro-element	MS (mg/l)	B5 (mg/l)
$MnSO_4.4H_2O$	22.3	-
$MnSO_4.H_2O$	-	10
$ZnSO_4.7H_2O$	8.6	2.0
H_3BO_3	6.2	3.0
KI	0.83	0.75
$CuSO_4.5H_2O$	0.025	0.025
$Na_2MoO_4.2H_2O$	0.25	0.25
$CoCl_2.6H_2O$	0.025	0.025
$FeSO_4.7H_2O$	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.2

Table 2.3: Composition of organics in MS (Murashige & Skoog 1962) and B5 (Gamborg *et al.* 1968) basal media.

Organics	MS (mg/l)	B5 (mg/l)
Thiamine. HCl	0.1	10
Pyridoxine HCl	0.5	1.0
Nicotinic acid	0.5	1.0
Myo-inositiol	100	100
Glycine	2.0	-

2.5. Collection of Plant material

Seeds of cotton cultivars LRK-516, LRA-5166, NHH-44, DCH-32, H-8 and DHY-286 were obtained from Maharashtra State Seed Corporation (MSSC), Akola, Maharashtra, India. The cultivar CNH-36 was obtained from Central Institute for Cotton Research (CICR), Nagpur, India (Fig. 2.1 A). Seeds were procured in the month of June - July and stored at room temperature for use throughout the year.

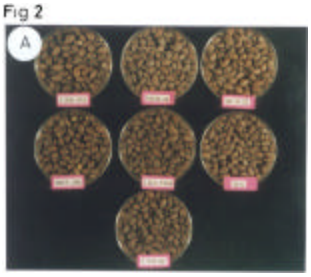


Fig.2.1

A. Seeds of cotton cultivars LRK-516,NHH-44,DCH-32, DHY-286, LRA-5166, H-8 and CNH-36

2.6. Preparation of plant material

2.6.1. Surface sterilization of seeds

Delinted seeds obtained from the above mentioned sources were washed with 1% vol/vol Labklin soap solution (S.D Fine Chem, India) for 5 min. Washed seeds were soaked in 10% vol/vol Savlon (a commercial antiseptic containing chlorhexidine gluconate 1.5% vol/vol and cetrimide 3% wt/vol; NR Jet enterprises, India) for another 5 min. Savlon was removed by rinsing the seeds three times with running tap water and then twice with glass-distilled water (1 min for each wash). The seeds were surface sterilized with 0.1% (wt/vol) mercuric chloride (Qualigens, India) for 10-12 min followed by 4-5 rinses of 1 min duration each with sterile glass-distilled water.

2.6.2. *In vitro* germination of seeds

For optimization of high frequency *in vitro* germination of seed, conditions like different basal media, presoaking treatment of sterilized seeds and incubation on moistened condition in petridishes were tested. Details of this have been described in chapter 3.

2.7. Inoculation

Aseptic explants derived from *in vitro* grown seedlings were inoculated in the media in a Laminar air-flow cabinet (Microfilt, India). Excision of the explants was carried out on sterile filter papers with the help of sterile scalpels and forceps. Scalpels and forceps were flame sterilized prior to inoculation and also in between the work by dipping in 70% rectified spirit. Surgical blades (No. 11 and No. 12) were used for excision of the explants. Sterile filter paper bridges (Whatman No.1) were used as supports for explants cultured in liquid media under static conditions.

All the experiments were repeated twice. The number of explants and replicates used in each experiment has been mentioned in material and methods of the respective chapters.

2.8. Statistical analysis

Standard deviations for the data were calculated and were analyzed statistically using one way or/ two-way analysis of variance (Snedecor & Cochran 1967).

2.9. Culture conditions

The cultures were incubated in culture room at 25 ± 2° C in dark or light (16 µE m^{-2} s^{-1}) and in Growth chamber (HERAEUS VÖTSCH, Germany) at a light intensity of (27 µE m^{-2} s^{-1}). The details of the incubation conditions have been mentioned in each section separately.

2.10. Histological studies

Histological analysis was carried out by fixing the plant specimens like cotyledonary node, embryo axis and scion/rootstock joint in 5-10 ml of FAA (Formalin: acetic acid: 70% ethanol by volume) (5: 5: 90) in 15 ml capacity screw capped vials (Borosil®, India) for 48 h at room temperature. Thereafter, the specimens were washed for 3-4 times with glass distilled water. Dehydration of the explants was carried out by passing them through t-butanol series (Sharma and Sharma 1980). This was followed by embedding in paraffin wax (melting point 58-60 ° C) (Merck, E. Merck India Ltd., Bombay, India). Sections of 10 µm thickness were cut using a rotary microtome (Reichert-Jung 2050 Supercut, Germany) and specimens were fixed on slides by mild heating. The sections were then passed through the xylene - alcohol series (Sharma and Sharma 1980) and stained with 1% Heidenhein's hematoxylin (wt/vol in distilled water, matured for one month in light) (Hi-Media Laboratories Pvt. Limited, Bombay) for one minute. The slides were counterstained with

1% eosin for two minute and mounted in DPX mountant. Histological sections mounted on slides were observed and photographed under a microscope (Docuval, Carl Zeiss, Germany).

2.11. Hardening of the plantlets

In vitro rooted shoots were carefully taken out of the test tubes and gently washed under tap water so as to remove the agar and medium sticking to it. The shoots were dipped in 1 % aqueous solution of bavistin®, a systemic fungicide (BASF, India) for 10-15 min and then washed with tap water. Thereafter the treated shoots were transferred in 8 cm earthen pots containing a mixture of autoclaved soil and sand (1:1) or soil: sand: compost (1:1:1). The pots were covered with polypropelene bags and kept in green house. The plants were watered once in a week. The top corners of polypropelene bags were cut after two weeks to gradually expose the plants to the outside environment. After 3-4 weeks, the polypropelene bags were completely removed.

2.12. Genetic Transformation

Details of materials & methods used for *Agrobacterium tumefaciens* and particle bombardment mediated transformations have been described in the chapters 5 and 6 respectively.

CHAPTER 3

IN VITRO INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION FROM COTYLEDONARY NODE AND ZYGOTIC EMBRYO AXIS EXPLANTS OF COTTON

3.1. Introduction

In vitro plant regeneration or micropropagation through tissue culture is an important step in the success of any crop improvement program. A rapid, simple and efficient plant regeneration protocol is also a major prerequisite for genetic transformation of crop plants.

Propagation under *in vitro* culture conditions can be achieved by using different plant parts such as axillary bud, shoot apex, hypocotyl, leaf and root etc. Plants can be propagated by tissue culture methods in three different ways: 1) by inducing the pre-existing shoot primordia to grow and multiply 2) by shoot morphogenesis, either directly from the explant or from unorganized tissues (direct or indirect caulogenesis), and by 3) somatic embryogenesis (direct or indirect embryogenesis).

The process of *in vitro* plant propagation from pre-existing meristems mainly consists of three steps: i) induction of shoot buds and their multiplication ii) elongation of shoot buds into shoots and iii) *in vitro* or *ex vitro* rooting of shoots to form plantlets. The process has several advantages like: propagation is simple, rapid and plants obtained are true to type, cultures can be initiated from very small segments from the mother plant, propagation in most of the species is possible throughout the year, greater degree of control over chemical, physical and environmental factors, possibilities of rejuvenation from mature tissues (Ahuja 1986).

3.1.1. *In vitro* plant regeneration in cotton

In vitro plant regeneration in cotton has been achieved by indirect somatic embryogenesis and through induction of shoots from pre-existing meristems.

Somatic embryogenesis via callus phase (indirect method) has been reported in several Coker and other varieties (Shoemaker *et al*. 1986; Trolinder & Goodin 1987; Gawel & Robacker 1990 a; Firoozabady & De Boer 1993; Kumar & Pental 1998 a b; Zhang *et al*. 2000 a). However, the method has been reported to generate undesirable somaclonal variations in cotton (Stelly *et al*. 1989; Firoozabady & De Boer 1993). An extensive seed-to-seed variability in *in vitro* regeneration has been observed among Coker lines (Trolinder & Xhixian 1989; Gawel & Robacker 1990 b). Maintenance of callus and cell cultures for longer periods often results in plants that are morphologically abnormal and

functionally sterile. Such variations pose a serious problem for maintenance of genetic uniformity in plants regenerated *in vitro* (Rajasekaran 1996 a). Due to these limitations, efforts have been made to regenerate plants from pre-existing meristems.

Several reports on plant regeneration via pre-existing meristems in cotton have been published (Table 1.10, Chapter 1). Chappel & Mauney (1967) were the first to report *in vitro* culture of excised meristems of *G. hirsutum* L. Although new leaf primordia could be initiated, the root system failed to develop. The next report was by Bajaj & Gill (1986) who could obtain plant regeneration by using shoot tips from field grown plants of *G. hirsutum* L.. Reports on plant regeneration in cotton using cotyledonary node as explant have been published (Agrawal *et al*. 1997; Gupta *et al*.1997; Hemphill *et al*. 1998; Hazra *et al*. 2000). Other explants such as embryo axis / shoot apex (Gould *et al*.1991; McCabe & Martinell 1993; Saeed *et al*.1997; Morre *et al*. 1998; Agrawal *et al*.1998; Zapata *et al*. 1999 b) have also been used. In all the reports on plant regeneration via embryo axis, except Morre *et al.* (1998), explants developed only single shoots. The embryo axis explant has many advantages as shoot regeneration is direct, relatively simple and is not prone to somaclonal variations and chromosomal abnormalities (Saeed *et al.* 1997).

Although work on *in vitro* plant regeneration via somatic embryogenesis has been reported mainly with American Coker cultivars, our preliminary efforts to extrapolate these results to Indian cultivars were not successful. Therefore, the main aim of this study has been to develop a plant regeneration protocol from explants having pre-existing meristems. The present chapter deals with the following two aspects:

1. Development of a plant regeneration protocol from cotyledonary node explants.
2. Establishment of a plant regeneration protocol from embryo axis explants.

3.2. Materials and methods

3.2.1. Plant material

Seeds of cotton cultivar LRK-516, LRA-5166, CNH-36, NHH-44, DCH-32, DHY-286 and H-8 were surface sterilized as described in chapter 2 (Materials and Methods).

3.2.2. Germination of seeds

Experiments with seven cultivars were carried out to obtain high frequency seed germination. Effect of basal medium and pre-soaking treatments on germination of seeds was tested.

3.2.2.1. Effect of basal media on germination of seeds

Four basal media combinations were used for seed germination: (1) Full strength of MS (Murshige & Skoog's) salts and vitamins, (2) half strength of MS salts and vitamins, (3) full strength of Gamborg's (B5) salts and vitamins and (4) MS salts and B5 vitamins. All the media were supplemented with sucrose (2%) and agar (0.55%). The pH of the media was adjusted to 5.8 before autoclaving. After surface sterilization, 100 seeds of each cultivar were directly inoculated on these four media in test tubes. These were incubated for 30 days at 25 $\pm$ 2°C under light intensity of 30 $\mu E\ m^{-2}\ s^{-1}$ with a 16 h photoperiod provided by cool white fluorescent tubes. Germination (seed with emergence of radicle) data was recorded after one week.

3.2.2.2. Effect of presoaking treatment on germination of seeds.

Seeds after surface sterilization were presoaked in 100 ml of sterile distilled water in a 250 ml flask for 1, 2, 4 h and 18 h. Thereafter, water was drained off and seeds were transferred to pre-sterilized petridishes containing a filter paper moistened with 1-2 ml of sterile distilled water. Petridishes were sealed with cling film (Flexo film wraps ltd, India) and incubated in dark at an ambient temperature for 48 h. Germination data was recorded after 2 days.

3.2.3. *In vitro* induction of multiple shoots

Experiments were carried out to study *in vitro* induction of multiple shoots in cotyledonary node and embryo axis explants. Cultivar LRK-516 was used for cotyledonary

node explants. Three cultivars LRK-516, NHH-44 and H-8 were used for embryo axis explants.

3.2.3.1. Explant preparation - cotyledonary nodes

Germinated seeds of cotton cultivar LRK-516 (Fig. 3.1A) were transferred to test tubes or in glass bottles containing 20 ml / 50 ml medium of MS salts and vitamins, sucrose (2%) and agar (0.55%) respectively. These were incubated for 5, 20 & 35 days at 25 $\pm$ 2°C under cool white fluorescent light at 30 μE m^{-2} s^{-1} with a 16 h photoperiod (Fig. 3.1B). Cotyledonary nodes were removed from 5, 20 and 35 day-old *in vitro* grown seedlings (Fig. 3.1C). The cotyledons and apical meristems were excised and discarded. Thus, each explant had two dormant axillary buds. These decapitated cotyledonary nodes were used as explants for induction of multiple shoots (Fig. 3.1D).

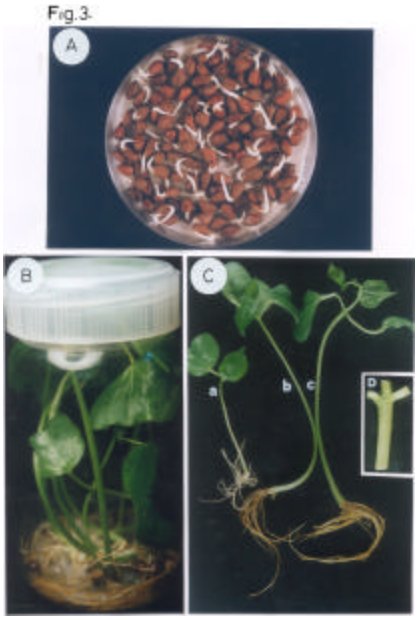


Fig. 3.1

A. Germinated seeds of cotton cultivar LRK-516 (after 48 hrs of incubation in dark).

B. Seedlings (35-days old) of cultivar LRK-516 in glass bottle.

C. Seedlings (a: 5-days; b:20-days and c:35-days old) of cultivar LRK-516 removed from test tubes.

D. Decapitated cotyledonary node explant excised from 35-day old seedling of cultivar LRK-516.

3.2.3.2 . Explant preparation - embryo axes

Embryo axes were excised aseptically (from germinated seeds of cultivars LRK-516, NHH-44 and H-8) and their radicles were discarded. The remaining 2 mm long embryo axis was used as an explant for induction of multiple shoots (Fig. 3. 2A, B).

Fig.3.2

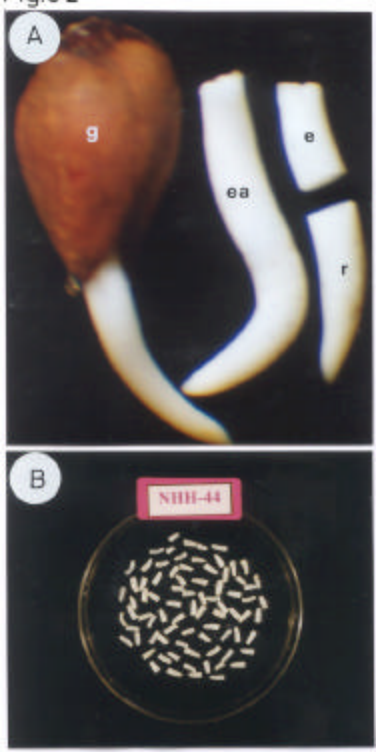


Fig. 3.2

A. Embryo axis explant excised from germinated seed: g=germinated seed, ea=embryo axis, e= explant, r = radicle.

B. Embryo axis explants excised from germinated seeds of NHH-44.

3.2.4. *In vitro* induction of multiple shoots from cotyledonary nodes

The influence of various phytohormones on morphogenetic response explants from 5, 20 & 35 day old seedlings and culture vessel on induction of multiple shoots from cotyledonary node was tested. Murashige and Skoog's (MS) salts and vitamins were used as basal medium for the whole experiment. All media were supplemented with 2 % sucrose and 0.55% agar and the pH was adjusted to 5.8 before autoclaving. All Cultures were incubated at 25 $\pm$ 2 ^{o}C under cool white fluorescent light at 30 μE m^{2} s^{-1} with a 16 h photoperiod.

3.2.4.1. Effect of different phytohormones on morphogenetic response

In a preliminary experiment, different phytohormones were tested to assess the morphogenetic response of the explant. Cotyledonary nodes excised from 20 day old seedlings were cultured in test tubes containing MS basal media supplemented with BAP (0.5 - 10 mg/l), kinetin (0.5 - 10 mg/l), TDZ (0.005 – 1.0 mg/l) and combinations of BAP (0.5 – 5 mg/l) and kinetin (0.5 - 5 mg/l). Cultures were incubated for 35 days under conditions mentioned as above. Fifty explants were cultured per treatment. At 35 days, the nature of explant's response was observed.

3.2.4.2. Effect of cytokinins and seedling age on induction of multiple shoots

In this experiment, cotyledonary nodes were excised from 5-, 20- and 35- day- old *in-vitro*-raised seedlings in test tubes. These explants were cultured on MS basal medium containing BAP (1.0 –2.5 mg/l) or kinetin (1.0 – 2.5 mg/l) or combinations of BAP and kinetin (0.5 –5 mg/l) in test tubes. Cultures were incubated under the conditions mentioned as above. At 35 days, only those explants which formed a minimum of one shoot on an average were considered as responded (expressed as % explant response), while formation of more than two shoots by an explant was considered as induction of multiple shoots.

3.2.4.3. Effect of culture vessel

In a separate experiment, the efficiency of multiple shoot formation from cotyledonary nodes excised from 35 days old seedlings raised in test tubes and glass bottles was evaluated. These explants were cultured on MS basal medium supplemented with BAP + kinetin (2.5 mg / l each) and were incubated as above.

3.2.5. *In vitro* induction of multiple shoots from embryo axes

In a preliminary set of experiment, influence of two basal media (MS salts and vitamins and MS salts+B5 vitamins) on sprouting response of embryo axes was tested. The selected basal medium (MS salts+B5 vitamins) was then supplemented with various phytohormones to determine the morphogenetic response. The selected phytohormones thereafter were evaluated for induction of multiple shoots from embryo axis of three cultivars. All media were supplemented with 2 % sucrose and 0.65 % agar (Hi-media, India) and the pH was adjusted to 5.8 before autoclaving. All cultures were incubated at 30 ^{o}C under cool white fluorescent light at 27 μE m^{-2} S^{-1} with a 16 h photoperiod.

3.2.5.1. Effect of basal medium on sprouting response

To test the sprouting response of embryo axes, 100 explants of each cultivar LRK-516, NHH-44 and H-8 were cultured in petridishes containing semisolid MS basal medium or MS salts+B5 vitamins. At the end of three weeks, the number of explants sprouted was scored.

3.2.5.2. Effect of various phytohormones on morphogenetic response

Embryo axis explants of NHH-44 were cultured in petridishes (85 mm x 15 mm) containing MS salts+B5 vitamins supplemented with various phytohormones such as BAP (0.005 - 3 mg/l.), Kinetin (0.005 – 3 mg/l), TDZ (0.001 – 0.2 mg/l), NAA (0.01 – 0.05 mg/l) either alone or in combinations of BAP (0.10 – 2 mg/l) and NAA (0.01 – 0.05 mg/l), TDZ (0.001 – 0.20 mg/l) and NAA (0.01 – 0.02 mg/l). After three weeks of incubation these were again transferred to fresh medium and were incubated for further 3 weeks. At the end of six weeks, the nature of explant's response was recorded.

3.2.5.3. Effect of BAP and NAA on induction of multiple shoots

Embryo axis explants of three cultivars (NHH-44, LRK-516, H-8) were cultured in petridishes (85mm x 15mm) containing MS salts+B5 vitamins supplemented with or without BAP (0.05 – 3 mg/l) or combinations of BAP (0.05 – 1 mg/l) and NAA (0.01 – 0.03 mg/l). Thirty explants (10 in each petridish) per treatment were cultured. After incubation for 3 weeks, the elongated shoot apices and elongated radicle ends in the embryo axis explants were excised and discarded. These decapitated explants were transferred to petridishes containing freshly prepared shoot induction media (as mentioned above) and were incubated for 3 weeks. Thereafter, these were transferred to 100 ml capacity Erlenmeyer flasks

containing 30 ml of medium and were incubated for further 6 weeks. At the end, the number of shoots in each explant was recorded. The explants (shoot bunches) were again transferred to 250 ml capacity Erlenmeyer flasks containing 100 ml of fresh medium and were incubated for 10 weeks. At the end of this period, the number of shoots formed per explant was recorded.

3.2.6. Elongation of shoots obtained from cotyledonary nodes

Multiple shoot bunches obtained from cotyledonary node explant were transferred to hormone-free MS basal medium containing 0.55% agar and 2% sucrose or to various MS basal media in test tubes supplemented with combinations of BAP (0.05 to 2.5 mg/l), kinetin (0.05 to 2.5 mg/l) and GA_3 (0.1-1.0 mg/l)

The multiple shoots were also cultured on liquid media of the same combinations (as above) in test tubes and supported with filter paper (Whatman No.1) bridges. Cultures were incubated under conditions as described above.

3.2.7. Elongation of shoots obtained from embryo axes

Elongation of shoots has occurred in the multiplication medium itself.

3.2.8. Rooting of *in vitro* shoots

3.2.8.1. Rooting of shoots derived from cotyledonary nodes

Elongated shoots (4 to 5 cm) derived from phytohormone free liquid medium were excised and cultured on half-strength MS basal medium (half concentration of MS salts and vitamins) containing 0.55% agar, 2% sucrose and with or without 0.05 or 0.1 mg/l NAA. Cultures were incubated under conditions as described in section 3.2.4.

3.2.8.2. Rooting of shoots derived from embryo axes

Shoots (3-4 cm) derived from multiple shoot bunches of embryo axis were excised and rooted on medium consisting of half strength MS basal medium supplemented with NAA (0.1 mg/l).

3.2.9. Transfer of plantlets to soil

Sixty rooted shoots obtained from multilple shoots of cotyledonary node and 40 rooted shoots from multiple masses of embryo axis were transferred to 8-cm pots containing

a 1:1 (vol/vol) mixture of soil and sand and kept under greenhouse conditions. Rooted shoots were hardened as described in chapter 2, section 2.11.

Plants (20 each from cotyledonary nodes and embryo axes) were transferred to field after 3-4 weeks of hardening in greenhouse.

All the above experiments were repeated twice

3.2.10. Histology

To confirm the origin of shoots, the cotyledonary nodes with multiple shoots were fixed in formalin: acetic acid: alcohol (5:5:90 vol/vol) for 48 h. Histology was carried out as described in the chapter no. 2 (Materials and Methods). Histology of embryo axis explant has been given in chapter 6.

3.3. Results and discussion

Results on seed germination of seven cultivars used in the entire course of study have been described in this chapter. Also results obtained on *in vitro* plant regeneration from cotyledonary node explants of cultivar LRK-516 and embryo axis explants from cultivars LRK-516, NHH-44, H-8 have been described.

3.3.1. Effect of basal media on germination of seeds

Out of four basal media formulations tested for seed germination, full strength MS salts and vitamins (i.e. basal medium) supported the maximum percentage of seed germination followed by Gamborg's (B5) salts and vitamins in all seven cotton cultivars after 7 days of incubation (Table 3.1).

Table 3.1: Effect of different basal media on germination of seeds (given as %).

Cultivars	Basal media			
	MS	Half MS	B5	MS salts + B5 vitamins
LRK – 516	27.33 ± 5.51	15.67 ± 3.79	21.67 ± 4.16	20.67 ± 2.08
LRA – 5166	23.00 ± 4.58	17.33 ± 2.89	20.33 ±2.52	19.00 ± 3.46
CNH – 36	23.33 ± 4.93	16.67 ± 4.16	20.67 ± 7.51	19.00 ± 2.00
NHH – 44	31.33 ± 3.51	20.33 ± 4.51	24.33 ± 4.16	21.67 ± 2.89
DCH – 32	29.00 ± 3.61	17.67 ± 2.52	22.00 ± 2.65	19.67 ± 4.16
DHY – 286	23.00 ± 2.65	14.33 ± 4.51	23.00 ± 5.29	21.33 ± 1.53
H – 8	26.33 ± 2.52	19.00 ± 1.73	21.67 ± 5.13	19.33 ± 2.89

Critical Difference among the treatments = 8.43

A marginal difference in germination percentage of seeds was noted when seeds were inoculated on B5 basal medium and MS salts+B5 vitamins. Among the seven cultivars tested, the highest percentage of seed germination was observed on full strength MS basal medium in cultivar NHH-44 (31.33%) followed by LRK –516 (27.33%), DCH-32 (29.00%), CNH-36 (23.33%), DHY-286 (23.00%) and LRA-5166 (23.00%). Similar to our study, germination of cottonseeds on agar solidified medium containing MS salts and B5

vitamins has earlier been achieved for Coker cultivar 310. However, the percentage of seed germination has not been mentioned (Finer 1988). Use of half strength MS basal medium for germination of cottonseeds has also been reported (Shoemaker *et al*. 1986). Recently, Zhang *et al*. (2000 a) have achieved seed germination by removing the kernels from the seeds and culturing them on a medium containing half strength MS salts+B5 vitamins. In our study, the low percentage of seed germination in all these cultivars could be due to the impurities in agar and / or the osmotic potential and turgor pressure developed in radicle cells due to high osmolarity.

3.3.2. Effect of presoaking treatment on germination of seeds

Cotton seeds presoaked for different periods such as 1, 2, 4 h. and 18 h in sterile double distilled water and incubated on sterile moistened filter paper in petridishes for 48 h resulted in a drastic improvement in percentage of seed germination compared to direct inoculation of seeds on agar solidified media. Maximum percentage of seed germination was observed in cultivar NHH-44 (81.00 ± 8.89), followed by LRK-516 (78.33 ± 7.09), LRA-5166 (74.67 ± 8.33), DHY-286 (63.67 ± 10.69), H-8 (59.67 ± 8.50), CNH-36 (56.33 ± 7.23) and DCH-32 (54.33 ±7.23) when presoaked for 1 h and incubated for 48 h (Table 3.2) on moistened filter paper in petridishes.

Table 3.2: Effect of presoaking treatments on germination of seeds (given as %).

Cultivars	Presoaking treatment			
	1 h	2 h	4 h	18 h
LRK – 516	78.33 ± 7.09	68.77 ± 8.74	54.00 ± 5.29	47.67 ± 7.37
LRA – 5166	74.67 ± 8.33	76.33 ± 7.02	62.00 ± 5.57	45.00 ± 6.56
CNH – 36	56.33 ± 7.23	48.67 ± 5.86	44.33 ± 3.51	40.33 ± 6.51
NHH – 44	81.00 ± 8.89	72.33 ± 5.51	55.67 ± 6.11	49.00 ± 7.00
DCH – 32	54.33 ± 5.86	43.33 ± 5.03	32.00 ± 4.00	23.67 ± 4.04
DHY – 286	63.67 ± 10.69	49.33 ± 7.64	45.00 ± 4.00	31.67 ± 4.04
H – 8	59.67 ± 8.50	51.67 ± 8.02	42.33 ± 4.16	28.67 ± 5.51

Critical Difference among the treatments = 14.37

The germination of cottonseeds on sterile moistened filter paper in petridishes has been reported earlier (Davidonis & Hamilton 1983; Trolinder & Goodin 1988). In a recent

study, Kumar *et al.* (1998 b) have obtained germination by removing the seed coat after soaking the seeds in sterile water for 4-5 hrs and on inoculation on half strength MS basal medium. This process of seed germination described in our study is simple and results in a high frequency seed germination where removal of seed coat is not required. Similar to our results, Hemphill *et al*. (1998) obtained seed germination by presoaking the seeds for 3 h and on incubation in sterile moistened condition at 30 ° C in dark.

3.3.3. *In vitro* induction of multiple shoots from cotyledonary node

This section describes results obtained on the effect of phytohormones on morphogenetic response, effect of cytokinins and seedling age and effect of culture vessel on induction of multiple shoots from cotyledonary node.

3.3.3.1. Effect of phytohormones on morphogenetic response

In the present study, the choice of MS basal medium is based on the earlier report on cotton (Bajaj & Gill 1986; Gould *et al*. 1991). On initial screening, cotyledonary node explants excised from 20 day old seedlings when cultured on MS basal medium supplemented with BAP (0.5 – 10.0 mg /l), kinetin (0.5-10 mg/l) and combination of BAP and kinetin (0.5 - 5.0 mg/l) resulted in shoot formation with different degree of response depending on the phytohormone tested (Table 3.3). BAP alone at 2.5 mg/l and in combination with kinetin (2.5 mg/l) resulted in 3-4 shoots/explant. Kinetin alone at 2.5 mg/l developed 2-3 shoots/explant after five weeks of incubation. Although the media containing lower concentrations of TDZ (0.005 – 0.05 mg/l) developed 2-3 shoots/explant, the percentage of response decreased with the increase of TDZ concentration in the media. All the TZD concentrations induced callus at the base of explants. TDZ levels 0.5 and 1.0 mg/l resulted in profuse callusing and shoots did not develop. (Fig.3.3A). The use of TDZ at low concentrations for micropropagation has been suggested (Lu 1993). Similar to present study, TDZ produced excessive callus and minimal shoot proliferation in peach whereas BAP supported the shoot proliferation (Zimmerman & Scorza 1992).

Fig.3.3

Fig. 3.3

A. Effect of TDZ on cotyledonary node explants of cultivar LRK–516 cultured on media supplemented with different concentrations of TDZ: a=0.005 mg/l, b=0.01 mg/l, c=0.05 mg/l, d=0.10 mg/l, e=0.5 mg/l, f=1.0 mg/l.

Table 3.3: Effect of phytohormones on morphogenetic response.

Phyto-Hormone	Conc. (mg/l)	Explant response %	Nature of response
BAP	0.0	76.0	1-2 shoots / Elongated
	0.5	82.0	1-2 shoots / Elongated
	1.0	92.0	2-3 shoots / Elongated
	2.5	88.0	2-3 shoots / Elongated
	5.0	58.0	2-3 shoots / Stunted
	10.0	16.0	Single shoot / Stunted
Kinetin	0.5	80.0	1-2 shoots / Elongated
	1.0	80.0	1-2 shoots / Elongated
	2.5	86.0	2-3 shoots / Elongated
	5.0	82.0	1-2 shoots / Stunted
	10.0	20.0	Single shoot / Stunted
BAP + Kinetin	0.5 + 0.5	88.0	2-3 shoots / Stunted
	1.0 + 1.0	92.0	2-3 shoots / Stunted
	2.5 + 2.5	52.0	3-4 shoots / Stunted
	5.0 + 5.0	46.0	1-2 shoots / Stunted
TDZ	0.005	56.0	2-3 shoots / Elongated / callus at the base
	0.01	52.0	2-3 shoots / Elongated / callus at the base
	0.05	40.0	2-3shoots / Stunted / callus at the base
	0.10	100.0	Profuse callus
	0.5	100.0	Profuse callus
	1.0	100.0	Profuse callus

3.3.3.2. Effect of cytokinins and seedling age on induction of multiple shoots

The induction of multiple shoots from explants varied with cytokinin type and concentration and was also influenced by the age of the seedlings (Table 3.4). Although each cotyledonary node explant had two dormant axillary buds at the time of inoculation, formation of an average of less than two shoots from 5-, 20- and 35-day-old seedlings was observed with most of the medium combinations (Table 3.4). This could be due to apical dominance of the growing shoots. A similar study on *Pisum sativum* reported development

of one or two shoots in cotyledonary node explants on medium devoid of phytohormones or supplemented with BAP (Jackson & Hobbs 1990).

MS basal medium without BAP or kinetin did not support the induction of multiple shoots (Table 3.4). BAP alone at a concentration of 1 or 2.5 mg/l induced multiple shoots in explants derived from 5- and 35- day-old seedlings, respectively. However, the percentage of explants forming multiple shoots was higher with 5- day- old seedlings than with 35- day-old seedlings (Table 3.4). Kinetin alone did not induce multiple shoot formation at any concentration or seedling age tested (Table 3.4).

The differential effect of various concentrations of BAP on the stimulation of shoot bud formation from cotyledonary nodes has already been reported for *Glycine* (Cheng *et al.*1980), *Pisum* (Jackson & Hobbs 1990) and *Phaseolus* (McClean & Grafton 1989). BAP was the most effective phytohormone in all these reports, indicating cytokinin specificity for multiple shoot induction in these tissues. In contrast to our study, Gupta *et al.* (1997) could not achieve shoot proliferation from cotyledonary node explants of cotton on culture in media supplemented with combinations of cytokinins (BAP, Kin and 2-ip). However, BAP alone at 5 mg/l supported maximum number of shoots/explant. It was observed that the number of multiple shoots/explant increased with the age of seedlings. In a recent study, Hemphill *et al*. (1998) have reported the use of BAP for induction of multiple shoot from pre-existing meristems of secondary leaf node, primary leaf node and cotyledonary node explants of cotton.

Explants from 35- day-old seedlings formed maximum shoots (4.7 shoots/explant) (Fig. 3.4A, B) on a medium supplemented with BAP + kinetin (2.5 mg/l each) followed by BAP + kinetin (1.0 mg/l each) with percentage response of explants being 100, 90 respectively. No further increase in the number of shoots could be observed with an increase in the concentration of cytokinins, indicating that a threshold was possibly achieved. The combined synergistic effect of the two cytokinins in the present study is similar to the results obtained for *Saccharum* spp., where a combination of BAP and kinetin induced a higher number of multiple shoots than in any other phytohormone treatments (Taylor & Dukic 1993). In another study, induction of multiple shoots could be achieved in *Vanilla walkeriae* with a treatment of BAP and kinetin in the presence of casein hydrolysate (Agrawal *et al.* 1992).

Table 3.4: Effect of cytokinins and seedling age on induction of multiple shoots.

Phyto-Hormone Conc.		Age of seedling					
		5 days		20 days		35 days	
BAP (mg/l)	Kinetin (mg/l)	Explant response %	No. of shoots/ Explant	Explant response %	No. of shoots/ Explant	Explant response %	No. of shoots/ Explant
0.0	0.0	100.0	1.05 ± 0.22	65.0	1.15 ± 0.37	80.0	1.12 ± 0.35
1.0	0.0	100.0	2.60 ± 0.84	95.0	1.94 ± 0.39	100.0	1.90 ± 0.87
2.5	0.0	84.0	1.14 ± 0.35	90.0	1.80 ± 0.40	60.0	2.33 ± 0.81
5.0	0.0	60.0	1.20 ± 0.56	64.0	2.06 ± 0.44	90.0	1.55 ± 0.52
0.0	1.0	100.0	1.14 ± 0.47	85.0	1.17 ± 0.38	90.0	1.33 ± 0.50
0.0	2.5	70.0	1.10 ± 0.55	87.0	1.31 ± 0.47	40.0	1.15 ± 0.48
0.0	5.0	86.0	1.12 ± 0.64	85.0	1.95 ± 0.49	70.0	1.11 ± 0.33
0.5	0.5	90.0	1.90 ± 0.56	90.0	2.05 ± 0.62	100.0	2.30 ± 0.48
1.0	1.0	100.0	2.30 ± 0.67	92.0	2.40 ± 0.59	90.0	2.80 ± 0.63
2.5	2.5	100.0	2.40 ± 0.51	48.0	2.08 ± 0.50	100.0	4.70 ± 0.67
5.0	5.0	60.0	1.60 ± 0.96	37.0	1.53 ± 0.51	40.0	2.04 ± 0.53

3.3.3.3. Effect of culture vessel

In our experiments, a marked difference was found in the number of multiple shoots obtained from explants derived from 35 day-old seedlings raised in test tubes and glass bottles (Table 3.5). Cotyledonary node explants excised from seedlings grown in glass bottles and cultured on medium supplemented with BAP and Kinetin (2.5 mg/l each) showed an increased induction of multiple shoots (8.3 shoots/explant) (Fig. 3.4C). In contrast, explants from seedlings raised in test tubes and cultured on the same medium gave 4.7 shoots/explant. This effect is probably due to the improved growth of seedlings with thick hypocotyls and dense green cotyledons in glass bottles compared to seedlings grown in test tubes, even though the height of seedlings in bottles and test tubes remained the same. The improved growth of seedlings in glass bottles may also be due to a larger amount of medium (50 ml compared to 20 ml in test tubes) and larger airspace (350 cm^3 compared to 30 cm^3 in test tubes) available to

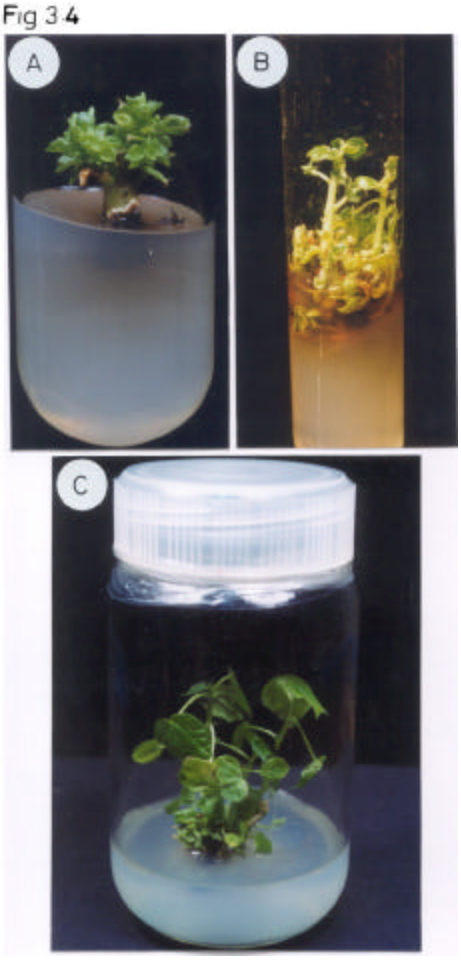


Fig. 3.4

A. Multiple shoots in cotyledonary node explant.

B. Proliferation of multiple shoots.

C. Multiple shoots in cotyledonary node explant (in glass bottle).

the seedlings, as the number of seeds germinated in test tubes and glass bottles was constant (2 seeds/vessel). The positive influence of larger culture vessel on shoot growth has

been reported earlier (McClelland & Smith 1990). Although, our study did not measure gaseous exchange in test tubes or bottles, the influence on shoot growth rate of the type of culture vessel and enclosures, due to varying amounts of ethylene / carbon di-oxide release, has been well documented (De Proft *et al.* 1985).

Table 3.5: Effect of culture vessel on induction of multiple shoots.

Phytohormones		Test tube		Glass bottle	
BAP (mg/l)	**Kinetin (mg/l)**	**Explant response %**	**Shoots/ explant (mean ± SD**	**Explant response %**	**Shoots/ explant (mean ± SD**
2.50	2.50	100.00	4.70 ± 0.67	100.00	8.30 ± 0.82

3.3.4. *In vitro* induction of multiple shoots from embryo axes

The following section describes results obtained on the effect of basal medium on sprouting response, effect of different phytohormones on morphogenetic response and, effect of BAP & NAA on induction of multiple shoots from embryo axis.

3.3.4.1. Effect of basal medium on sprouting response

Experiments carried out to assess the effect of two basal media formulations (MS salts and vitamins and MS salts+B5 vitamins) on sprouting response of embryo axes varied with media formulations and the cultivars tested. The medium containing MS salts+B5 vitamins supported the maximum sprouting response (Fig.3.5A) in all three cultivars compared to MS salts and vitamins (Table 3.6). In this medium, the maximum percentage of sprouting response was observed in cultivar NHH-44 (96.00 %) followed by LRK-516 (94.50 %) and H-8 (80.00 %) (Table 3.6). Therefore, MS salts+B5 vitamins as basal medium were used in all our further experiments with embryo axis. The use of MS salts and modifications in the constituents of vitamins in basal media meant for *in vitro* plant regeneration in cotton from pre-existing meristems have been reported by several workers (Gould *et al.* 1991; Gupta *et al.* 1997; Hemphill *et al.* 1998; Zapata *et al.* 1999 b). Zapata

et al. (1999 b) could achieve maximum sprouting (58%) from shoot apices of cotton by culturing the explants on MS salts+modified B5 vitamins without phytohormone.

Table 3.6: Effect of basal media on sprouting response.

Basal medium	Sprouting response (%)		
	NHH-44	LRK-516	H-8
MS salts & vitamins	88.50 ± 7.78	86.50 ± 4.95	76.50 ± 6.36
MS salts & B5 vitamins	96.00 ± 2.83	94.50 ± 2.12	80.00 ± 7.07

3.3.4.2. Effect of various phytohormones on morphogenetic response

In an initial experiment, embryo axis explants cultured on media containing MS salts+B5 vitamins and supplemented in a wide range of BAP, Kinetin, TDZ, NAA either alone or in combinations exhibited different morphogenetic response (Table 3.7). Of all the phytohormones tested, BAP alone (0.05 and 0.1) or in combination with NAA (0.01-0.03) supported formation of 2-3 shoots /explant. BAP concentrations higher than 0.1 mg/l resulted in a decreased number of shoots and in the percentage of response. Zapata *et al*. (1999 b) had a similar observations with cotton shoot apices where plant regeneration was suppressed with higher concentrations of BAP.

Kinetin at all concentrations except 0.1 mg/l supported formation of only single shoots. Shoots developed at kinetin concentrations (0.3- 1.0 mg/l) remained stunted. At higher levels of kinetin (3.0 mg/l), explants did not respond and turned brown (Table 3.7). In a similar study, Gould *et al*. (1991) had observed blackening of tissues at 1.0 mg/l of kinetin.

Explants cultured on the medium containing TDZ alone at 0.005 mg/l resulted in 1-2 stunted shoots, developed callus and roots at radicle end. TDZ concentrations from 0.05 – 0.2 mg/l induced profuse callusing and did not develop shoots. The basal medium

supplemented with NAA alone at 0.01 and 0.02 mg/l supported elongation of shoots and simultaneous induction of roots in 90 and 94% of explants respectively (Table 3.7).

The basal medium containing TDZ (0.005 mg/l) and NAA (0.01 mg/l) resulted in 1-2 stunted shoots/explant and induced callus and roots at the explant base. Higher concentrations of TDZ and NAA resulted in profuse callusing all over the explant.

From the above pilot study it can be concluded that BAP alone or BAP+NAA are the most potential phytohormones for induction of multiple shoots from embryo axis explants. Therefore, further experiments were carried out to optimize the concentrations of these two phytohormones for induction of multiple shoots from embryo axis explants of three cotton cultivars viz. NHH-44, LRK-516 and H-8.

Table 3.7: Effect of different phytohormones on morphogenetic response.

Phyto-hormone	Conc. (mg/l)	% of explant response	Nature of response
BAP	0.005	84.00	Single shoot / Elongated
	0.01	94.00	Single shoot/ Elongated
	0.05	90.00	2-3 shoots / Elongated
	0.10	70.00	2-3 shoots / Elongated
	0.3	52.00	1-2 shoots / Elongated
	0.5	46.00	Single shoot / Stunted
	1.0	40.00	Single shoot / Stunted
	3.0	32.00	Single shoot / Stunted
Kinetin	0.005	92.00	Single shoot / Elongated
	0.01	80.00	Single shoot / Elongated
	0.05	74.00	Single shoot / Elongated
	0.10	62.00	1-2 shoots / Stunted
	0.3	44.00	Single shoot / Stunted
	0.5	32.00	Single shoot / Stunted
	1.0	18.00	Single shoot / Stunted
	3.0	00.00	Turned brown
TDZ	0.001	60.00	Single shoot / Elongated
	0.005	50.00	1-2 shoots / Stunted / Callus at the base
	0.01	42.00	1-2 shoots / Stunted
	0.05	100.00	Callus
	0.1	100.00	Profuse callus

	0.2	100.00	Profuse callus
NAA	0.01	90.00	Single shoot / Roots / Elongated
	0.02	94.00	Single shoot / Roots / Elongated
	0.05	70.00	Roots / Profuse callus

Cotnd.

Phyto-hormone	Conc. (mg/l)	% of explant response	Nature of response
TDZ + NAA	0.001 + 0.01	70.00	Single shoot / roots / Elongated shoot and root / callus
	0.005 + 0.01	52.00	1-2 shoots / roots / Stunted shoot /Callus
	0.10 + 0.01	100.00	Profuse callus
	0.20 + 0.02	100.00	Profuse callus
BAP + NAA	0.50 + 0.01	70.00	2-3 shoots / roots / Elongated shoot and root
	0.10 + 0.02	86.00	2-3 shoots / Elongated shoot and root
	0.50 + 0.03	40.00	2-3 shoot / Elongated shoot
	1.00 + 0.03	36.00	Single shoot / Stunted shoot
	1.00 + 0.05	24.00	Single shoot / Stunted shoot
	2.00 + 0.05	12.00	Single shoot / Stunted shoot

3.3.4.3. Effect of BAP and NAA on induction of multiple shoots

Different concentrations of BAP, BAP+NAA induced shoots (Fig. 3.5B) in embryo axes varying from 1 to 5.95 per explant depending upon the cultivar and the medium composition (Table 3.8). Even though, the maximum number of shoots in all three cultivars was observed on the medium containing BAP (0.1 mg/l) + NAA (0.02 mg/l), the number of multiples formed per explant varied among the three cultivars (Table 3.8). The highest number of multiple shoots was observed in cultivar NHH-44 (5.95) followed by LRK-516 (4.44) and H-8 (3.88). Further proliferation of these shoots was observed on transfer of shoot masses to the fresh medium and on incubation for 10 weeks. This resulted in more

than two fold increase in multiple shoots. The highest number of shoots was recorded in cultivar NHH-44 (13.08) (Fig. 3.5C) followed by H-8 (9.72) and LRK-516 (7.84).

Fig 3.5

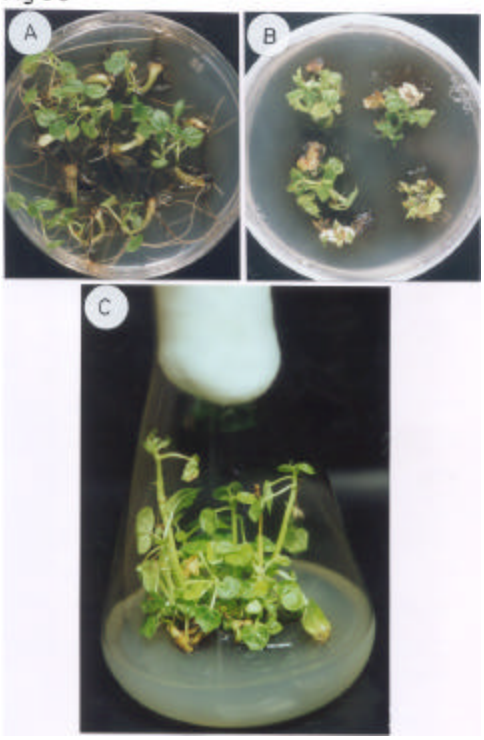


Fig. 3.5

A. Development of shoot and root in embryo axis explants after 4 weeks of incubation.

B. Induction of multiple shoots in embryo axis.

C. Multiple shoots in embryo axis explants of cultivar NHH-44 after five months of incubation.

In our earlier experience with cotyledonary nodes (Agrawal *et al*. 1997), it was observed that non-decapitated explants did not form multiple shoots and is the reason for excising and discarding the elongated shoot apices after an initial incubation of explants for 3 weeks. This is in contrast to the findings in an earlier report on Argentine cotton cultivar, where multiple shoot induction in similar explants could be achieved without decapitation and with the use of BAP alone (3 mg/l) (Morre *et al*. 1998). The maximum number of multiple shoots formed however was restricted to an average of 3.4 and also the report is limited only to one genotype. In the present study, we found that for Indian cultivars of cotton, decapitation of explants and their culturing on a medium with BAP (0.1 mg/l) + NAA (0. 02 mg/l) was essential to maximize the induction of multiple shoots. Inclusion of BAP alone (3 mg/l) in the medium did not induce shoot multiples. In a similar experiment, Hemphill *et al*. (1998), obtained only stunted shoots from embryo axes on a medium containing BAP alone (2.25 mg/l). The effect of decapitation of shoot apex from cotyledonary nodes and phytohormones on induction of multiple shoots has been discussed earlier (Agrawal *et al.* 1997).

The second best response in terms of number of shoots/explant was observed on medium supplemented with BAP (0.05 mg/l)+NAA (0.01 mg/l) in all three cultivars. Though the maximum number of shoots was observed in cultivar NHH-44 (4.34) followed by LRK-516 (2.98) and H-8 (2.86), the percentage of response was maximum in cultivar LRK-516 (80.0) followed by NHH-44 (76.7) and H-8 (63.3). The medium supplemented with BAP (0.50 mg/l)+NAA (0.03 mg/l) did not support the production of multiple shoots in all three cultivars.

Of all the BAP concentrations tested, 0.10 mg/l promoted the maximum number of shoots in all three cultivars (Table 3.8). BAP concentrations higher than 0.1 mg/l did not induce multiple shoots.

In general, cotton is considered to be recalcitrant in *in vitro* culture system and its response is restricted to only a few genotypes (Trolinder & Goodin 1987; Trolinder & Xhixian 1989; Cousins *et al*. 1991). Though plant regeneration in cotton has been achieved from embryo axis or shoot apices, explants formed only single shoots (Gould *et al*. 1991; McCabe & Martinell 1993; Saeed *et al*. 1997; Agrawal *et al*. 1998; Zapata *et al*. 1999 b). Prior to our report, only Morre *et al.* (1998) have induced multiple shoots from embryo axis explant of an Argentine cotton cultivar cv. Guazuncho II.

Table 3.8. Effect of BAP and NAA on induction of shoots.

Phytohormones		Cultivars					
		NHH-44		LRK-516		H-8	
BAP (mg/l)	NAA (mg/l)	Explant response %	No. of shoots/ explant	Explant response %	No. of shoots/ explant	Explant response %	No. of shoots/ explant
0.00	0.00	98.3	1.90 ± 0.69	96.7	1.21 ± 0.58	78.3	1.41 ± 0.49
0.05	-	91.7	3.38 ± 0.85	80.0	2.45 ± 0.74	65.0	2.02 ± 0.73
0.10	-	76.7	3.96 ± 0.69	76.7	2.91 ± 0.85	56.7	2.85 ± 0.90
0.50	-	50.0	1.54 ± 0.51	43.0	1.46 ± 0.51	40.0	1.42 ± 0.59
1.00	-	40.0	1.16 ± 0.37	30.0	1.11 ± 0.33	31.7	1.16 ± 0.49
3.00	-	30.0	1.00 ± 0.00	15.0	1.0 ± 0.00	25.0	1.06 ± 0.19
0.05	0.01	76.7	4.34 ± 0.89	80.0	2.98 ± 0.77	63.3	2.86 ± 0.90
0.10	0.02	81.7	5.95 ± 1.16	78.3	4.44 ± 0.95	71.7	3.88 ± 1.11
0.50	0.03	43.3	1.80 ± 0.69	55.0	1.38 ± 0.49	35.0	1.47 ± 0.70
1.00	0.03	35.0	1.26 ± 0.42	20.0	1.17 ± 0.41	26.7	1.25 ± 0.48

Critical Difference for percentage of explants response is 12.12
Critical Difference for average number of shoots/ explant is 0.47.

3.3.5. Elongation of shoots obtained from cotyledonary nodes

The multiple shoots obtained on various shoot induction media did not elongate on the same media. Therefore, the multiple shoots obtained from 35 day-old seedlings on MS basal medium supplemented with BAP and kinetin (2.5 mg/l each) were transferred as a mass to test tubes containing various media combinations (Table 3.9). On an average, elongation of 2.97 shoots per culture tube with an average length of 4 cm (Fig. 3.6A) from the mass of multiple shoots was obtained in liquid MS basal medium without phytohormone (Table 3.9). Elongation of shoots with an average length of 3.5 cm was also observed on liquid MS medium supplemented with BAP (0.50 mg/l) + kinetin (0.50 mg/l) + GA_3 (1.00 mg/l). Similarly, liquid MS basal media supplemented with BAP + kinetin + GA_3 in a few other combinations also supported elongation of shoots (Table 3.9).

In general, semisolid MS basal media with BAP (0.10) + kinetin (0.10) + GA_3 (0.50) mg/l and their higher concentrations supported elongation of only 1.1 shoots on an average, except the medium containing BAP (1.0) + GA_3 (0.50) mg/l, which resulted in elongation of 1.9 shoots on an average (Table 3.9). This leads to the conclusion that media without phytohormones or lower concentrations of phytohormones favoured only the elongation of shoots.

In general, liquid media were more effective than agar media (Table 3.9). Elongation of shoots on hormone free medium has been obtained in *Clianthus formosus* (Taji & Williams 1989), *Leucopogon obtectus* (Bunn *et al.* 1989) and a hybrid larch (Brassard *et al.* 1996). In several other studies, with *Pinus brutia* (Abdullah *et al.* 1987) and *Picea abies* (Ewald & Suss 1993), shoot elongation was enhanced by reducing the cytokinin concentration in the medium. As in the present study, cytokinins have often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Brassard *et al.* 1996).

Table 3.9: Effect of cytokinins and media with and without agar on elongation of multiple shoots.

Phytorhormones			Semisolid media			Liquid media with filter paper support		
BAP (mg/l)	Kinetin (mg/l)	GA_3 (mg/l)	% of shoot masses showing elongation	Shoots elongated / shoot mass (Mean ± SD)	Length of elongated shoot (cm) (Mean ± SD)	% of shoot masses showing elongation	Shoots elongated / shoot mass (Mean ± SD)	Length of elongated shoot (cm) (Mean ± SD)
0.00	0.00	0.00	90.00	1.96 ± 0.51	3.25 ± 0.54	93.00	2.97 ± 0.74	4.0 ± 0.44
0.05	0.05	0.10	53.00	1.93 ± 0.57	2.00 ± 0.66	63.00	1.94 ± 0.52	2.5 ± 0.52
0.10	0.10	0.50	46.00	1.07 ± 0.26	1.50 ± 0.47	60.00	1.94 ± 0.63	2.5 ± 0.40
0.50	0.50	1.00	50.00	1.06 ± 0.25	1.50 ± 0.52	93.00	1.96 ± 0.83	3.5 ± 0.53
1.00	0.00	0.50	33.00	1.90 ± 0.31	1.75 ± 0.42	40.00	1.91 ± 0.51	2.5 ± 0.70
1.00	1.00	0.50	33.00	1.10 ± 0.31	1.00 ± 0.40	33.00	0.90 ± 0.31	2.0 ± 0.77
2.50	2.50	0.00	20.00	0.83 ± 0.40	0.56 ± 0.23	26.00	1.12 ± 0.35	1.5 ± 0.70

3.3.6. Rooting of *in vitro* shoots derived from cotyledonary nodes

Rooting of shoots occurred after 12-15 days on half-strength MS basal medium without growth regulators or with NAA (0.05 or 0.1 mg/l). Formation of roots (Fig. 3.6B) was observed in 75% of shoots on half-strength MS basal medium without supplements and 88.33% or 91.66 % rooting of shoots with 0.05 or 0.1 mg/l NAA respectively (Table 3.10). In addition, adventitious root formation was more profound in half-strength MS basal medium supplemented with 0.05 or 0.1 mg/l NAA than in medium without auxin. Use of low salt MS medium for rooting of *in vitro* shoots has been reported for *Philodendron* spp. (Maene & Debergh 1985). Contrary to the present study, Saeed *et al*. (1997) could not achieve *in vitro* rooting of cotton shoots derived from shoot tips when cultured on half strength and full strength MS basal medium. However, rooting could be induced on MS basal medium supplemented with NAA (2.68 mM) + kinetin (0.46 mM). In an another study, Gould *et al* (1991) could achieve rooting of shoots (30 %) by application of rooting agent in a potting mixture. Recently, Hemphill *et al.* (1998) accomplished rooting of *in vitro* shoots by dipping shoot base in a rooting agent (rootone) and on direct transfer to soil.

Table 3.10: Effect of NAA on rooting of *in vitro* derived shoots from cotyledonary node.

Medium	No. of shoots rooted (Mean ± SD)	Percentage of rooting	No. of hardened plants transferred to soil
Half strength MS	45 ± 2.64	75.00	60
Half strength MS + NAA (0.05 mg/l)	53 ± 3.21	88.33	
Half strength MS + NAA (0.10 mg/l)	55 ± 2.08	91.66	

3.3.7. Rooting of shoots de rived from embryo axes

The elongated shoots were rooted (Fig. 3.6C) on a medium containing half strength of MS salts+vitamins and supplemented with NAA (0.1 mg/l) (medium already standardized for rooting of shoots derived from cotyledonary node). The maximum percentage of rooting (85 %) was observed in cultivar LRK-516 followed by NHH-44 (82.50) and H-8 (80%).

Fig. 3.6

A. Elongated shoots on filter paper bridge in liquid medium.

B. *In vitro* rooted shoot (derived from cotyledonary node) in half strength of MS basal medium.

C. Rooted shoot (derived from embryo axis) on half strength MS basal medium supplemented with NAA (0.1 mg/l).

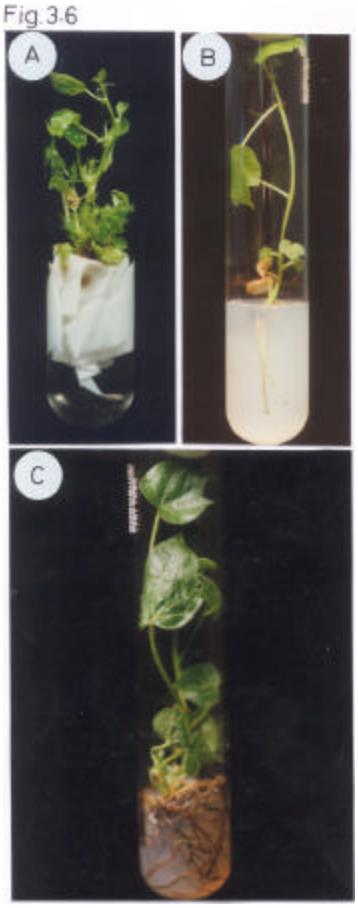


Fig. 3.6

A. Elongated shoots on filter paper bridge in liquid medium.

B. *In vitro* rooted shoot (derived from cotyledonary node) in half strength of MS basal medium.

C. Rooted shoot (derived from embryo axis) on half strength MS basal medium supplemented with NAA (0.1 mg/l).

3.3.8. Transfer of plantlets to soil

Out of sixty rooted shoots (derived from cotyledonary nodes) transferred to pots containing a soil mixture (soil: sand, 1:1) under greenhouse conditions, 95% survived after 2 months (Fig. 3.7A). Normal boll formation (arrow) (Fig.3.7B) was observed in all the tissue culture raised plants 4 months after transfer to field.

Survival of the rooted shoots (derived from embryo axes) was found to be 92% after hardening in greenhouse (Fig. 3.8A). All the plants grew to maturity and developed normal bolls 4 moths after transfer to field (Fig. 3.8B).

3.3.9. Histology

The histology of cotyledonary node (longitudinal sections) at 35 days of development confirms the direct multiplication of shoot bud initials (arrows) and shoot (s) (Fig.3.9A).

Fig. 3.7

A. Hardened tissue culture plants in earthen pots kept in the greenhouse.

B. Mature tissue culture plant of cultivar LRK-516 in field.

Fig 3.8

Fig. 3.8

A. Hardened tissue culture plants of cultivar NHH-44 in pots.

B. Mature tissue culture cotton plant in field showing squares (arrow), and dehisced boll (arrow).

Fig. 3.9

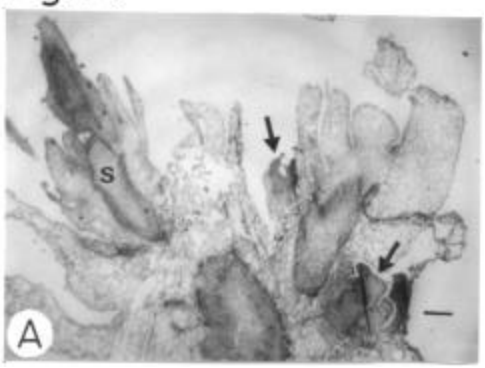


Fig. 3.9

A. Longitudinal section of a cotyledonary node region showing shoot bud initials (arrows), shoot (s) (bar=242 μm).

3.4. Conclusion

In the present chapter, efforts were made to develop plant regeneration protocols from cotyledonary nodes of cultivar LRK-516 and embryo axes of three cultivars viz. NHH-44, LRK-516 and H-8. Induction of multiple shoots could be achieved from cotyledonary nodes devoid of cotyledons and apical meristems. Explants from 35-day-old seedlings yielded the maximum number of shoots (4.7 shoots/explant) on Murashige and Skoog's (MS) basal medium supplemented with BAP and kinetin (2.5 mg/l each). Explants from 35-day-old seedlings raised in glass bottles produced a higher number of multiple shoots (8.3 shoots/explant) than those grown in glass tubes and cultured on the same shoot induction medium indicating a positive influence of culture vessel. Elongation of multiple shoots was obtained on liquid or agar MS basal medium without phytohormones. The best rooting response *in vitro* shoots was achieved on half-strength semisolid MS basal medium with NAA (0.1 mg/l). Survival of tissue culture plantlets was 95% after hardening under greenhouse conditions.

Also, induction of multiple shoots, its proliferation and regeneration of whole plants from embryo axes of three cultivars of cotton was achieved. The maximum number of multiple shoots in all three cultivars could be induced on a medium containing BAP (0.1) and NAA (0.02 mg/l). Further shoot proliferation was achieved on transfer of multiple shoot bunches to the fresh medium and on incubation for 10 weeks. Rooted shoots survived (92%) after hardening in greenhouse and grew to maturity (100%) after transfer to field.

The present investigations have significance, since study of this nature on Indian cotton cultivars has not been reported so far. The results will be of advantage in the ongoing efforts in genetic transformation of Indian cotton cultivars by particle bombardment and *Agrobacterium* mediated methods.

Plant regeneration from cotyledonary node explants has been published as a paper entitled "*In vitro* induction of multiple shoots and plant regeneration in cotton (*Gossypium hisutum* L.)" by Agrawal DC, Banerjee AK, Kolala RR, Dhage AB, Kulkarni AV, Nalawade SM, Hazra S and KV Krishnamurthy, Plant Cell Reports, Vol 16: 647-652, 1997

A part of this work has been filed as an Indian Patent (Ref. No. NF-31/2000 dated 28-3-2000) entitled " A tissue culture medium formulation useful for induction and proliferation of multiple shoots in excised embryo axes of cotton" by Agrawal DC, Banerjee AK, Nalawade SM, Hazra S and KV Krishnamurthy.

A manuscript entitled "Multiple shoot induction and plant regeneration from embryonic axes of Indian cultivers of cotton" by Banerjee AK, Agrawal DC, Nalawade SM and KV Krishnamurthy is also under preparation.

CHAPTER 4

EFFECT OF ANTIBIOTICS ON SHOOT GROWTH OF ZYGOTIC EMBRYO AXIS EXPALNTS OF COTTON

4.1. Introduction

The term 'antibiotic' appeared as early as 1928 in the French microbiological report on antibiosis (Burkholder 1952). An organism protects itself against its enemies by producing specific toxic substances, which interfere with the metabolism of other organism to such an extent that they are either killed or prevented from multiplying. These specific toxic substances are referred to as antibiotics and the phenomenon is termed as antibiosis (Salle 1954). However, the word antibiotics in its present restrictive meaning –"a chemical substance derived from microorganisms which has the capacity of inhibiting growth and even destroying, other microorganisms in dilute solutions" – was introduced by Selman Waksman in 1942. This definition of antibiotic might be broadened to include all microbial compounds which are able to selectively affect various biochemical growth processes at minimal concentration in humans, animals, plants or microorganisms (Vandamme 1984). Antibiotics are produced by all classes of organisms such as bacteria, yeasts, molds, plants and animals. Since 1945, thousands of different antibiotics produced by fungi, actinomycetes, or unicellular bacteria have been isolated and characterized. The first chemotherapeutically effective antibiotic, penicillin was discovered by Alexander Fleming in 1929. The organism which produced penicillin was *Penicillium notatum*. The most important antibiotics are all produced from actinomycetes and other bacteria and from moulds.

4.1.1. Nature of antibiotics

Antibiotics have extensive application in the treatment of infectious disease of man, animals and to a smaller extent plants. They differ widely in their physical and chemical properties. Some show close similarity, whereas others differ considerably. They act also in various ways. Some antibiotics interfere with growth and cell division of organisms, still others interfere with the utilization of essential metabolites. Antibiotics may be germicidal in high concentrations and bacteriostatic in less concentrated preparations (Salle 1954). They are generally categorized by similarities in chemical structures. Perhaps the best known group of antibiotics are the aminoglycosides, quinones, lactones and peptides (Young *et al.* 1984)

4.1.2. Antibiotics used in the present study, their properties and producer microorganisms

Four antibiotics (Cefotaxime, carbenicillin, streptomycin and chloramphenicol) (Table 4.1) were used in the present study. Among them, cefotaxime-sodium salt (cephalosporin) and carbenic illin-disodium salt belongs to the β-lactam group of antibiotics and these bind to the 50S subunits of the prokaryote ribosome thereby interfering with protein synthesis. Both antibiotics inhibit cell wall synthesis in dividing bacterial cells resulting in their lysis. Streptomycin is an aminoglycoside group of antibiotic, which binds to 30S subunits of the bacterial cells and inhibits protein synthesis (Young *et al*. 1984). Streptomycin was the first example of an antibiotic, which possessed a broad spectrum activity against Gram positive and Gram negative bacteria (Stanier 1987). Chloramphenicol is also a β-lactam group of antibiotic. Cefotaxime has a broader antimicrobial spectrum and higher pharmacological activity than carbenicillin (Wise *et al.* 1978; Doerr *et al.* 1980).

Table 4.1: The antibiotics used in the present study and their producer microorganisms.

Antibiotic	Produced by
Cefotaxime (Cephalosporin)	*Acremonium chrysogenum* (*Cephalosporium acremonium*)
Cabenicillin	A semisynthetic penicillin
Streptomycin	*Streptomyces griseus*
Chloramphenicol	*Streptomyces venezuelae*

4.1.3. Use of antibiotics in agriculture

Apart from various medicinal applications, antibiotics have also been used in agriculture especially as antihelmintics, herbicides, insecticides, miticides, hormonal compounds and food preservatives. Antibiotic streptomycin, chloramphenicol and oxytetracycline have been reported to control bacterial diseases whereas cycloheximide and griseofulvin have been used to combat fungal diseases of plants. Hygromycin is used as antihelmintic. Herbicidin, piericidin and tetranactin have been used as herbicides, insecticides

and miticides respectively. Gibberellins isolated from *Gibberella fujikuroi* have been used as plant hormones. Natamycin and nisin are used as food preservatives (Vandamme 1984).

4.1.4. Role of antibiotics in plant tissue cultures

In general, antibiotics are phytotoxic in plant tissue cultures (Young *et al*. 1984; Waldenmaier *et al*. 1986), however, certain antibiotics have shown to stimulate plant growth and development. In its first report, Holford (1992) showed stimulation in growth and induction of roots in *Vinca rosea* due to additions of penicillin, penicillin G and streptomycin. In several other plants such as in sugarbeet (Catlin 1990), wheat (Mathias & Boyd 1986) and barley (Mathias & Mukasa 1987), the positive responses of antibiotics have been observed. The use of antibiotics in plant tissue culture has become more common now due to application of antibiotic resistant genes as selectable markers and co-cultivation of various explants with *Agrobacterium tumefaciens* in transformation experiments (Herrera-Estrella *et al*. 1983; Llyod *et al*. 1986; Umbeck *et al*. 1987; Zhan *et al*. 1997). Selection of transformed cells is critical for recovery of transgenic plants, and this has been an obstacle in some crops like grapes that are sensitive to kanamycin (Gray & Meredith 1992). Typically the optimum concentration of the selective agent should be one that prevents regeneration without being too toxic to the target explant (Yepes & Aldwinckle 1994).

Using plant cell cultures of *Nicotiana plumbaginfolia*, Pollock *et al*. (1983) examined relative toxicities of a wide range of antibiotics and observed that cephalosporins provided broad spectrum anti-microbial activity without significant toxicity to plant cells and also recommended it as one of the group of antibiotics of choice to plant cells. Cefotaxime, a cephalosporin, is commonly used to eliminate *Agrobacterium* after co-cultivation of plant tissues for transformation. Besides anti-microbial activity, cefotaxime has earlier been reported to influence morphogenesis in barley (Mathias & Boyd 1986) and wheat cultures (Borrelli *et al*. 1992). In an another study, cefotaxime and carbenicillin have been reported to be the best antibiotics for selective killing of *Agrobacterium* when co-cultivated with sugar beet tissues. Streptomycin and chloramphenicol both inhibit the growth of sugarbeet and carrot tissues at concentrations slightly lower than required for inhibition of plant regeneration (Okkels & Pedersen 1988). The influence of kanamycin in regeneration of shoots in tobacco and carrot tissues has also been reported (Owens 1979).

Both carbenicillin and cefotaxime have been used earlier to eliminate *Agrobacterium tumefaciens* cells in transformation experiments with cotton (Umbeck *et al.* 1987; Firoozabady *et al*.1987). However, effects of both these antibiotics on cotton cultures have not been reported so far.

In our transformation experiments with cotton embryo-axes, cefotaxime was incorporated in the medium to eliminate *Agrobacterium*. However, it was observed that when embryo-axes (which were not co-cultivated with *Agrobacterium*) were inoculated onto the medium with cefotaxime as controls, it resulted in higher frequency of shoot development (Chapter 5).

This observation has lead us to examine the effects of cefotaxime and three other antibiotics on embryo axis of cotton cultivars and their possible role in high frequency development of shoots and to determine the usefulness of such information in transformation studies with embryo-axes of different cultivars of cotton. The other three antibiotics (Streptomycin, carbenicillin and chlroramphenicol) were also incorporated in the study since these are some of the most commonly used antibiotics in plant cell cultures and routinely used for elimination of *Agrobacterium.*

4.2. Materials and methods

4.2.1. Plant material

Delinted seeds of six cultivars of cotton (*G. hirsutum* L. cv. LRK-516; LRA-5166; NHH-44; DCH-32; CNH-36; DHY-286) were surface sterilized and disinfected by the method described in chapter 2. The disinfected seeds were soaked in sterile water for 1 h prior to inoculation on sterile filter paper moistened with 1-2 ml of sterile distilled water in sterilized petri-dishes. The seeds were kept in dark for 48 h for germination.

4.2.2. Explant preparation

Under aseptic conditions, embryo-axes were excised from germinated seeds, their radicles were dissected out and discarded while the remaining 2 mm long embryo-axes were split longitudinally into two halves. These two halves of embryo axes were considered as two explants.

4.2.3. Preparation of antibiotic stock solution

Sources of antibiotics (cefotaxime, chloramphenicol, carbenicillin and streptomycin) used in the present study have been mentioned in chapter 2 (Materials and Methods). Since antibiotics carbenicillin, chloramphenicol and streptomycin procured were in non sterile form, these were dissolved in sterile double distilled water and the solutions were filter sterilized through a 0.22 µm membrane (Millipore). Cefotaxime procured was in sterile form.Therefore, it was aseptically dissolved in sterile double distilled water and the solution was used without filter sterilization. Aliquots of stock solutions of all these antibiotics were dispensed individually into 1.5 capacity eppendorf tubes and stored at – 20° C till further use.

4.2.4. Culture medium and conditions

The excised explants were cultured on a medium containing Murashige and Skoog's (1962) salts, B5 vitamins (Gamborg *et al*.1968) and supplemented with different concentrations of cefotaxime (100–1000 mg/l), carbenicillin (100-1000 mg/l), chloramphenicol (1-10 mg/l) and streptomycin (100-1000mg/l) individually. Sucrose (2%) and agar (0.55%) were added to all the media and pH was adjusted to 5.8 before autoclaving. Antibiotic solutions were aseptically added to the autoclaved media before the media were poured into the pre-sterilized petridishes of 55 mm diameter. The explants

inoculated in petridishes were kept at 25 ± 2° C under 16 h photoperiod of white fluorescent light intensity of 30 uE $m^{-2}s^{-1}$. Each dish contained 12 explants and each treatment had eight replicates. The data was analyzed using Student `T' test. All the experiments were repeated twice.

4.2.5. Effect of Cefotaxime on the frequency of shoot development, length of shoots and fresh weight of explants

Different concentrations of cefotaxime (100, 250, 500, 750 and 1000 mg/l) were tested and observations like frequency of shoot development, length of shoots and fresh weight of explants were recorded after 30 days of incubation.

4.2.6. Effect of Cefotaxime on induction of shoots in prolonged incubation of embryo axis

The effect of cefotaxime concentrations (100, 250, 500 and 750 mg/l found to be optimum for cultivars DHY-286, DCH-32, LRA-5166, NHH-44 respectively) were further tested for their effects on prolonged incubation. Ten explants per petridish (85 mm x 15 mm) in four replicates were kept for each cultivar and per each treatment. Cultures were incubated for a period of three months instead of one month as in the earlier experiments. Cultures were transferred to fresh medium after six weeks of incubation. After three months, data on response of explants and the number of shoots per explant developed were recorded.

4.2.7. Effect of carbanecillin and streptomycin on the frequency of shoot development

The effect of carbenicillin and streptomycin on the frequency of shoot development was studied only with cotton cultivar NHH-44, since out of six cultivars tested, it yielded the highest frequency of shoot development with cefotaxime.

Different concentrations of carbanecillin and streptomycin (100, 250, 500, 750 and 1000 mg/l) were tested for the frequency of shoot development in cultivar NHH-44. The observations were recorded after 30 days of incubation.

4.2.8. Effect of chloramphenicol on the frequency of shoot development

The effect of chloramphenicol on the frequency of shoot development was studied only with cotton cultivar NHH-44 with the same justification as given in 4.2.7.

Effect of different concentrations of chloramphenicol (1, 2, 5, 7 and 10 mg/l) was tested for the frequency of shoot development in cultivar NHH-44. The observations were recorded after 30 days of incubation.

4.3. Results and discussion

Six cultivars used in the present study were selected since these are widely grown in southern states of India. Split embryo axis explant was chosen for the present investigations since it was used for our *Agrobacterium* mediated transformation experiments (chapter 5). The choice of basal medium used is based on our earlier experience with embryo axis explant used for multiple shoot induction studies. Results on the effect of these antibiotics on the frequency of shoot development and certain growth parameters of Indian cultivars have been presented and discussed in the following sections:

4.3.1. Effect of cefotaxime on frequency of shoot development, length of shoots and fresh weight of explants

4.3.1.1. Frequency of shoot development

An influence of cefotaxime on the frequency of shoot development was observed in all of the six cultivars of cotton. The frequencies of shoot development in embryo-axes varied depending upon the cultivar and the concentration of cefotaxime added in the medium (Fig. 4.1A). Cefotaxime at varying levels in different cultivars induced higher frequencies of shoot development as compared to controls. However in LRK-516, it had an inhibitory effect at all the levels tested (Fig.4.1A).

Embryo axis explants cultured on medium devoid of cefotaxime (i.e. controls) resulted in 49.11, 37.50, 42.36, 31.25, 38.89 and 34.73% of shoot development in LRK-516, LRA-5166, NHH-44, DCH-32, CNH-36 and DHY-286 respectively (Fig.4.1A). In this medium, explants elongated and developed into shoots and roots (Fig. 4.2A). Cefotaxime at a concentration of 750 mg/l yielded the maximum frequency of shoot development (59.37% and 44.04%) in cultivars NHH-44 (Fig 4.1 A; Fig. 4.2B) and CNH-36 respectively. In case of cultivar DHY-286, the maximum shoot development (44.52%) was recorded at 100 mg/l of cefotaxime. On further increase of this concentration, a gradual decrease in frequency of shoot development was observed. Application of cefotaxime at 500 and 250 mg/l resulted in 49.02 and 46.43% of shoot development in LRA-5166 and DCH-32 respectively. In cultivars LRA-5166, NHH-44, DCH-32 and CNH-36, cefotaxime at the highest concentration (1000 mg/l) also increased the frequency of shoot development compared to the controls (Fig. 4.1A).

4.3.1.2. Length of shoots

The effect of cefotaxime on shoot length varied depending upon the cultivar and concentration applied (Fig. 4.1B). A positive influence of cefotaxime on shoot length was observed in all the cultivars except LRK-516. Cefotaxime at all concentrations tested inhibited the shoot length in cultivar LRK-516. In case of DCH-32, a gradual increase in shoot length was observed on increase of cefotaxime concentrations from 100 – 500 mg/l while higher levels had an inhibitory effect. The trend was just the reverse in case of cultivar LRA-5166. In another cultivar NHH-44, cefotaxime at a concentration of 750 mg/l yielded maximum increase in shoot length (1.70 ± 0.16 cm).

4.3.1.3. Fresh weight of explants

The most striking effect of cefotaxime on fresh weight of explants was observed in cultivar LRK-516. Concentrations of cefotaxime (500 and 750 mg/l) induced profuse callus formation in the explants (Fig. 4.2C) and resulted in an increase in fresh weight (0.44 and 0.43 gm respectively) (Fig. 4.1C). Callusing was observed at the cut ends of the explants after two weeks of incubation. Shoots developed from these explants were stunted probably due to excessive callusing of the explants. Cefotaxime had an inhibitory effect on fresh weight of explants of DCH-32 at all the concentrations tested. In case of cultivar DHY-286, cefotaxime had very marginal effect on fresh weight of explants compared to controls (Fig. 4.1C).

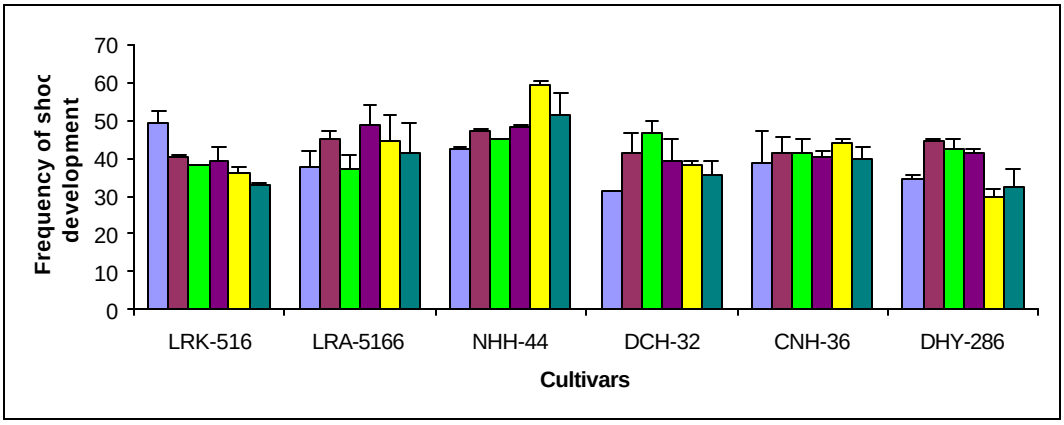
In the present study, cefotaxime influenced the frequency of shoot development, length of shoots and fresh weight of explants. However, the precise pattern of response varied between the cultivars. Besides controlling bacterial contamination in cultures, cefotaxime has growth promoting effects on embryo-axes of cotton cultivars. However, addition of optimum concentration of cefotaxime in the medium is important since at supra-optimal levels, inhibition of growth and profuse callusing has been observed.

Improvement in growth and development of plant tissues by application of certain antibiotics has earlier been reported. Cefotaxime at low concentrations have earlier been found to stimulate plant regeneration in carrot and sugarbeet (Okkels & Pedersen 1988). In a study on wheat (*Triticum aestivum* L.), incorporation of 60 to 100 mg/l of cefotaxime in culture medium enhanced callus production from immature embryos and also increased production of organogenic calli (Mathias & Boyd 1986). The stimulatory effect of

cefotaxime on morphogenesis has also been observed in pear (Predieri *et al*. 1989). In another study on four cultivars of barley (*Hordeum vulgare* L.), cefotaxime increased callus growth and/or regeneration frequencies of plants (Mathias & Mukasa 1987). It was also observed that the inclusion of cefotaxime in the protoplast culture medium was essential for inducing cell division in isolated *Passiflora* protoplasts (Vaz d'Utra *et al.*1993).

Though there are no conclusive reports which hitherto clarify the effect of cefotaxime on plant cells (Vaz d'Utra *et al*.1993), the growth regulatory effect of cefotaxime has been explained by Mathias & Mukasa (1987). They postulated that active molecule of cefotaxime mimics a plant growth regulator. Another possibility suggested that degradation of cefotaxime by plant esterases might generate metabolites, which account for the growth regulator like activity (Mathias & Boyd, 1986).

A



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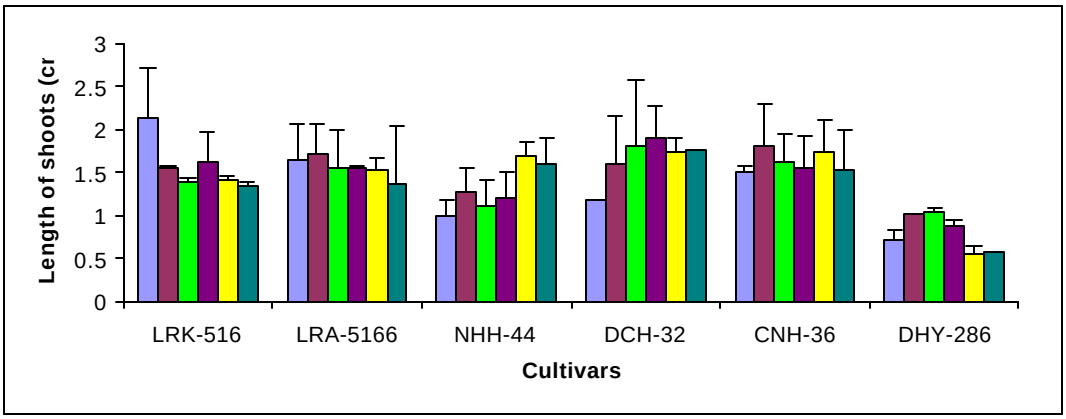


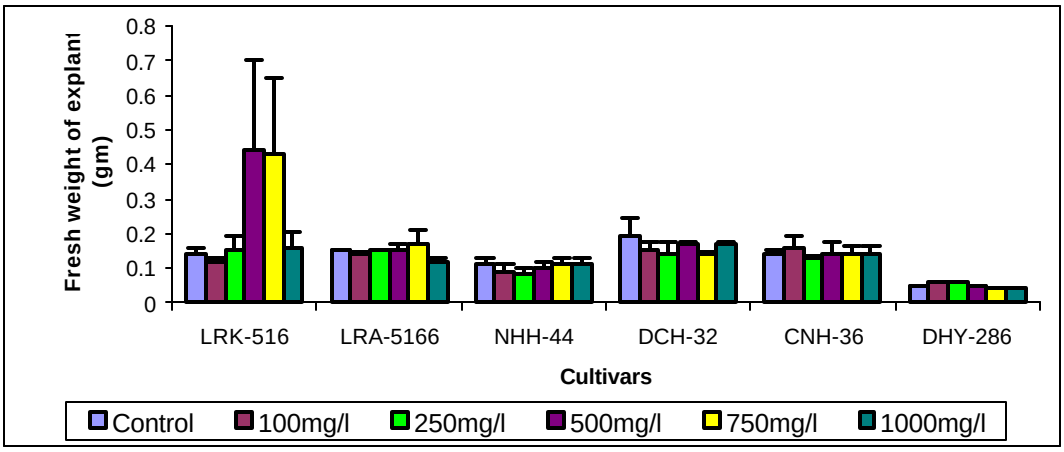


Fig. 4.1

Effect of cefotaxime on excised embryo-axes of six cultivars of cotton:

A. Frequency of shoot development, B. Length of shoots C. Fresh weight of explants.

Fig 4 2

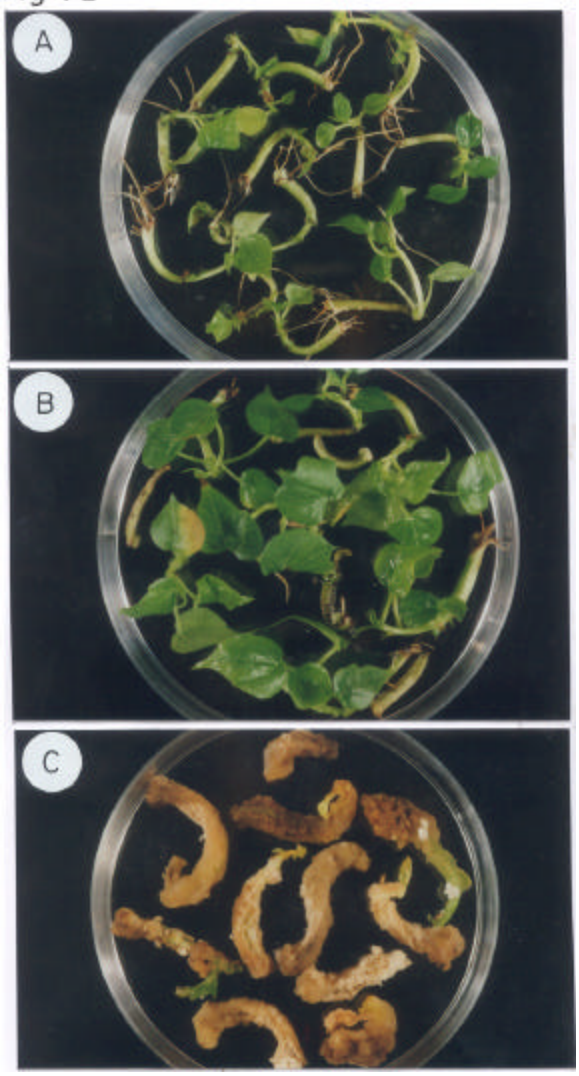


Fig. 4.2

A. Embryo axis explants of cultivar NHH-44 after one month of culture in MS medium devoid of cefotaxime.

B. Embryo axis explants of cultivar NHH-44 after one month of culture in MS medium supplemented with cefotaxime (750 mg/l).

C. Callus formation in embryo axis explants of cultivar LRK- 516 cultured in MS medium supplemented with cefotaxime (500 mg/l).

4.3.2. Effect of Cefotaxime on induction of shoots on prolonged incubation of embryo axis in culture medium

This study was carried out with four cultivars i.e. NHH-44, LRA-5166, DCH-32 and DHY-286 since cefotaxime had an inhibitory effect on cultivar LRK-516 and viable seeds of cultivar CNH-36 were not available at the time of this investigation. Embryo axes cultured on optimum concentrations of cefotaxime (750, 500, 250 and 100 mg/l for culitivars NHH-44, LRA-5166, DCH-32 and DHY-286 respectively**)** and incubated for a period of three months with a intermittent transfer onto fresh medium resulted in an increase in the number of shoots per explant in all the four cultivars as compared to controls. Among these, NHH-44 (Fig. 4.3A) formed the maximum number of shoots per explant (2.57 ± 0.66) followed by LRA–5166 (2.26 ± 0.57), DCH-32 (2.24 ± 0.77) and DHY-286 (1.83 ± 0.63) (Table 4.2). On prolonged incubation, explants cultured on media with or without cefotaxime developed profuse roots.

Fig. 4.3

Fig. 4.3

A. Embryo axis explants of cultivar NHH-44 after three months of culture in MS medium supplemented with cefotaxime (750 mg/l).

Table 4.2: Effect of cefotaxime concentrations on development of shoots after three months of incubation of embryo axis.

Cultivar	Media	Percentage of response	No. of Shoots/ explant (Mean ± SE)
NHH -44	MS – CT	41.25^{c}	$1.46^{d} \pm 0.63$
	MS + CT (750mg/l)	53.75^{a}	$2.57^{a} \pm 0.66$
LRA-5166	MS – CT	36.25^{d}	$1.35^{de} \pm 0.49$
	MS + CT (500 mg/l)	43.75^{c}	$2.26^{b} \pm 0.57$
DCH-32	MS – CT	33.75^{d}	$1.34^{de} \pm 0.49$
	MS + CT (250 mg/l)	46.25^{b}	$2.24^{b} \pm 0.77$
DHY-286	MS – CT	36.25^{d}	$1.38^{d} \pm 0.50$
	MS + CT (100 mg/l)	43.75^{c}	$1.83^{c} \pm 0.63$

MS – Murashige and Skoog's salts with B5 vitamins; CT- Cefotaxime.

Means followed by similar superscript do not differ significantly at 95% level of confidence.

4.3.3. Effect of carbenicillin and streptomycin on the frequency of shoot development

The effect of carbenicillin and streptomycin on the frequency of shoot development was studied only with cotton cultivar NHH-44, since out of six cultivars tested, it yielded the highest frequency of shoot development (59.37%) with cefotaxime. Carbenicillin at 500 mg/l level had the highest frequency of shoot development (57.81%) (Fig. 4.4A) followed by 250 mg/l (56.76%) and 100 mg/l (52.08%) compared to controls (44.78%) (Fig. 4.5A). Carbenicillin at levels higher than 500 mg/l reduced the frequency of shoot development. At higher levels, swelling of the explants and poor growth of shoots was observed. Earlier, Okkels & Pedersen (1988) had studied the effect of carbenicillin on cultures of sugarbeet and carrot. They observed that carbenicillin at low concentrations inhibited the callus growth while relatively higher concentrations (>1000 mg/l) had no effect on plant regeneration. In a separate report, Mathias & Boyd (1986) studied the difference in shoot formation from calli of wheat when cultured on media supplemented with autoclaved or unautoclaved carbenicillin. They found that the autoclaved carbenicillin had inhibitory effect on shoot production compared to unautoclaved carbenicillin.

Streptomycin at all levels (100-1000mg/l) had an inhibitory effect on shoot development in NHH-44. Medium devoid of streptomycin resulted in the maximum frequency of shoot development (45.30%). Streptomycin at 100, 250 and 500 mg/l levels supported 18.22, 13.01 and 5.20% of shoots respectively (Fig. 4.5A). Shoots at these levels became chlorotic, did not elongate and developed only 2-3 leaves (Fig. 4.4B). Streptomycin at levels higher than 500 mg/l could not develop any shoots in embryo axis explants. Bastian *et al* .(1983), earlier has observed the phytotoxic effect of streptomycin on tissue cultures of *Ficus benjamina* and *Cordyline terminalis*. Contrary to their findings, Owens (1979) has reported a positive influence of streptomycin in regeneration of shoots in tobacco leaf sections. Streptomycin is an aminoglycoside group of antibiotics which binds to 30S subunit of bacteria and inhibits protein synthesis. The phytotoxic effect of streptomycin observed in the present study could be due to the inhibition of protein synthesis in

chloroplasts and mitochondria, which, like bacteria, contain 30S and 50S ribosomal subunits (Young *et al.* 1984).

4.3.4. Effect of chloramphenicol on the frequency of shoot development

Compared to cefotaxime, carbenicillin and streptomycin, chloramphenicol has earlier been used for cell cultures at lower concentrations (1.0-10.0 mg/l) because of its toxic effects at higher concentrations (Lurquin & Kleinhofs 1982; Pollock *et al*.1983**).** In the present study, chloramphenicol at levels of 1 and 2 mg/l had a positive influence on frequency of shoot development in cultivar NHH-44 and resulted in 52.08 and 55.20% frequency of shoot development respectively compared to controls (43.74%) (Fig. 4.5B). Shoots developed on medium supplemented with chloramphenicol were healthy and normal (Fig. 4.4C). Chloramphenicol concentrations higher than 2 mg/l were inhibitory and decreased the shoot development. Earlier, Pollock *et al*. (1983) has observed that chloramphenicol at a level above 1 mg/l is toxic to protoplast derived cells of *N. plumbaginifoliai.* In an another study, it was observed that growth of the *Helianthus tuberosus* explants was inhibited when cultured at a level of 50 mg/l of chloramphenicol (Phillips *et al.* 1981).

Fig. 4 4

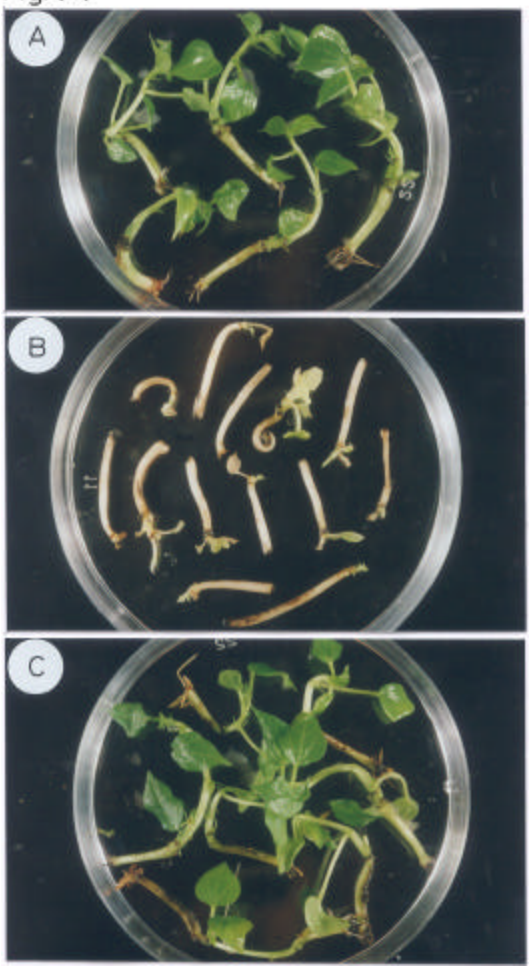


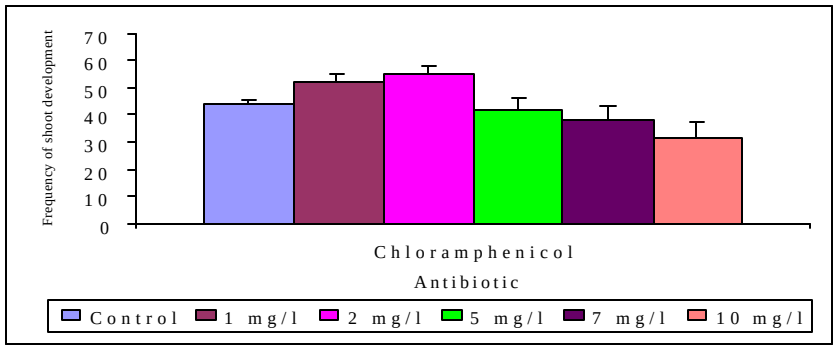
Fig. 4.4

A. Embryo axis explants of cultivar NHH-44 after one month of culture in MS medium supplemented with carbenicillin (500 mg/l).

B. Embryo axis explants of cultivar NHH-44 after one month of culture in MS medium supplemented with streptomycin (100 mg/l).

C. Embryo axis explants of cultivar NHH-44 after one month of culture in MS medium supplemented with Chloramphenicol (2 mg/l).

A - B



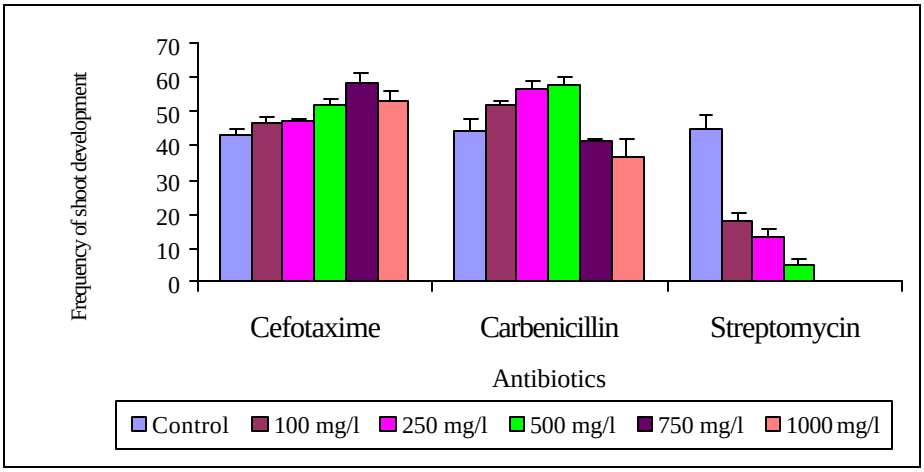


Fig. 4.5

A. Effect of cefotaxime, carbenicillin and streptomycin on the frequency of shoot development from excised embryo axes of cotton cultivar NHH-44.

B. Effect of chloramphenicol on the frequency of shoot development from excised embryo axes of cotton cultivar NHH-44.

4.4. Conclusion

Of the four antibiotics tested in the present study, barring streptomycin, other three antibiotics cefotaxime, carbenicillin and chlroramphenicol at certain concentrations had a positive influence on embryo axis explants of cotton cultivars. The effect of these antibiotics depended on its concentration and the cultivar tested. Streptomycin at all concentrations had a negative effect on the frequency of shoot development in cotton cultivar NHH-44. The results obtained in the present study demonstrate that out of four antibiotics tested, cefotaxime is the best antibiotic for cotton embryo axes. The data collected can be used to determine the optimum concentration of cefotaxime required by different cultivars to obtain maximum response of shoot development. The information collected can be utilized gainfully in *Agrobacterium* mediated transformation with embryo-axes and to recover a large number of transformants through high frequency development of shoots during post co-cultivation studies.

Part of this work has been filed as an Indian Patent (Ref. No. 381/97 dated 18-6-1998) and also published as a paper entitled "Effect of cefotaxime on the growth of excised embryo axes of six cultivars of cotton (*Gossypium hirsutum* L.) by Agrawal DC, Banerjee AK, Kedari PH, Jacob S, Hazra S and KV Krishnamurthy. Journal of Plant Physiology, Vol. 152, 580-582 (1998).

CHAPTER 5

AGROBACTERIUM TUMEFACIENS MEDIATED TRANSFORMATION STUDIES IN COTTON

5.1. Introduction

Though conventional plant breeding methods have resulted in a spectacular improvement in crop production, there are strong pressures for further improvement in crop quality and quantity due to explosion in population, social demands, health requirements, environmental stresses and ecological considerations (Kung 1993). Conventional plant breeding techniques have limitations as these depend on sexual compatibility and often take 10-15 years to release a new variety due to extensive backcrossing (Pauls 1995). These limitations have stimulated the development of more advanced technologies like genetic transformation of plants.

Genetic transformation can be defined as the transfer of foreign genes isolated from plants, viruses, bacteria or animals into a new genetic background. In plants, successful genetic transformation requires the production of normal, fertile plants, which express the newly inserted genes. The process of genetic transformation involves several distinct stages, namely insertion, integration, expression and inheritance of the new DNA. Process of gene insertion can involve the use of bacterial (*Agrobacterium* species) or viral vectors or direct gene transfer methods (Webb & Morris 1994).

Genetic engineering has allowed explosive expansion of our understanding in the field of plant biology and provides us with the technology to modify and improve crop plants. A remarkable progress has been made in the development of gene transfer technologies (Gasser & Fraley 1989) which ultimately have resulted in production of a large number of transgenic plants both in dicots and monocots. Potential benefits from these transgenic plants include higher yield, enhanced nutritional values, reduction in pesticides and fertilizer use and improved control of soil and water pollutants. Some of the important characters like resistance to herbicide (Smith 1994), disease (Smith 1994), insect (Perlak *et al*. 1990), high protein content (Habben & Larkins 1995), cold tolerance (Georges *et al*. 1990), fruit quality (Fray & Grierson 1993), biodegradable plastics (Poirier *et al*. 1995), antibodies and vaccines (Mason *et al*. 1992) etc. have been incorporated in the genetically engineered plants

5.1.1. History of genetic transformation

It took more than 2000 years to detect the causal principle of the crown gall disease after it was first described by Aristotle's and Theophrastus (Siemens & Schieder 1996). Smith & Townsend (1907) were the first to report that *Agrobacterium tumefaciens* is the causative

agent of the widespread neoplastic plant disease crown gall. Since then a large number of scientists throughout the world have focused their research to understand the molecular mechanism of crown gall induction. The soil bacterium *A. tumefaciens* and *A. rhizogenes* are considered as natural genetic engineers due to their ability to transfer and integrate DNA into plant genomes through a unique integrative gene transfer mechanism (Jouanin *et al.* 1993).

It was only in 1983 that scientist inserted the first foreign genes into *Petunia* and tobacco (Kung 1984). *Agrobacterium*-mediated gene transfer became the method of choice due to convenience and high probability of single copy integration. Independently, several transgenic tobacco plants were produced to express foreign genes engineered by the *Agrobacterium tumefaciens* vectors (Murai *et al*.1983; Horsch *et al*. 1984; De Block *et al*. 1984). Initially successes in genetic transformation were limited to the species of *Solanaceae* especially tobacco (*N. tavacum* L.). However, this changed the situation dramatically in late 80's and early 90's and resulted in transformation of a wide range of plants for agronomically important traits (Songstad *et al*.1995) using genetically engineered avirulent strains of *Agrobacterium* as vectors (Herrera-Estrella *et al.* 1983).

Since the initial successes in the *Agrobacterium* mediated transformation were mostly confined to dicotyledonous plants, concerted efforts were made to look for alternative methods of gene transfer. Method of direct gene delivery into protoplasts was the next development in genetic transformation (Draper 1982), Further many more techniques such as macroinjection (Zhou *et.al.* 1983, 1988), soaking pollen in DNA solution (Ohta 1986), pollen transformation via pollentube pathway (Luo & Wu 1988), microinjection (Neuhaus & Spangenberg 1990**),** silicone carbide fibres (Kaeppler *et al* 1990), electroporation (DeKeyser *et al*.1990), sonication (Joersbo & Brunstedt 1990), electrophoresis (Griesbach & Hammond 1993), laser mediated gene transfer (Guo *et al*. 1995) have been developed. However, none of these approaches has, so far, been developed into a reproducible universal gene transfer technique (Potrykus 1995)

The next breakthrough in genetic transformation was the development of biolistic (Particle bombardment) transformation approach (Klein *et al*. 1987; Sanford 1988). Details of this methodology have been described in the chapter 6.

5.1.2. *Agrobacterium* mediated gene transfer technique:

Agrobacterium is a gram-negative, soil-dwelling bacterium, which infects plant cells near wounds, usually at the junction between the root and stem (crown) in a wide range of plant species. *Agrobacterium*–mediated gene transfer involves incubation of cells or tissues with the bacterium (cocultivation), followed by regeneration of plants from the transformed cells. For plant species that are readily amenable to tissue culture, *Agrobacterium* mediated gene transfer, the first widely adopted methods of developing transgenic plants, remains the most popular technique. Probably the greatest advantage of the system is that it offers the potential to generate transgenic cells at relatively high frequency, without a significant reduction in plant regeneration rates. The system is simple, inexpensive and in many cases efficient. Moreover the DNA transferred to the plant genome is defined, it does not normally undergo any major rearrangements and it integrates into the genome as a single copy (Walden & Wingender 1995).

Agrobacterium tumefaciens possess a tumor inducing (Ti) plasmid responsible for the tumor formation (Zupan & Zambryski 1995) whereas *A. rhizogenes* possess a root inducing (Ri) plasmid which is responsible for DNA transfer and the resulting hairy root formation (Tepfer 1984). During infection, the bacterium transfers a small section of its own genetic material (T-DNA) into the genome of the host plant's cell (Zambryski 1992). Once inserted, the bacterial genes are expressed by infected cells of that plant. During the infection process, first the plant cell begins to proliferate and form tumors and then synthesize an arginine derivative called opine. The opine synthesized is usually nopaline or octopine depending on the strain involved. These opines are catabolized and used as energy sources by the infecting bacteria. By understanding and manipulating this process of infection or transformation, scientists have been able to harness these powerful and sophisticated vectors to transfer specific cloned genes of major importance.

Initially, monocotyledons were considered outside the host range of *Agrobacterium*. However, advances in understanding of the biology of the infection process, availability of gene promoters suitable to monocotyledons (Wilmink *et al.* 1995) as well as selectable markers have improved transformation of monocotyledons (Smith & Hood 1995). Transgenic plants of rice (Hiei *et al*. 1994) and maize (Ritchie *et al*. 1993) have been produced via *Agrobacterium*-mediated transformation.

So far, more than hundred plant species have been transformed by *Agrobacterium*-mediated transformation technique (Siemens & Schieder 1996). However, success of *Agrobacterium*–mediated transformation depends on the cultivar (Robinson & Firoozabady

1993), the choice of explants (Robinson & Firoozabady 1993; Jenes *et al.* 1993); the delivery system, the *Agrobacterium* strain (Gelvin & Liu 1994); the conditions of cocultivation, the selection method and the mode of plant regeneration. *Agrobacterium* cocultivation has been successfully used for the transformation of leaves, roots, hypocotyls, petioles, cotyledons (Zambryski 1992; Hooykaas & Beijersbergen 1994), pollen-derived embryos (Sangwan *et al.* 1993), seeds (Feldmann & Marks1987) and even plants (Chang *et al.* 1994).

T-DNA of *Agrobacterium* is a small section of the plasmid DNA, about 23 kb in size, which makes up about 10% of the Ti or Ri plasmids. This stretch of DNA is flanked by 25bp repeated sequences, which are recognized by the endonucleases encoded by the vir genes. Within the T-DNA, two distinct regions TL and TR have been identified. The T-DNA of nopaline strains can integrate as a single segment, whereas octopine strains frequently integrate as two segments, TL and TR. TL carries the genes controlling auxin and cytokinin biosynthesis and is always present when tumors are formed. Failure of TR to integrate results in the loss of opine biosynthesis (Webb & Morris 1992). The *vir* (virulence) region of Ti plasmid contain the genes which mediate the process of T-DNA transfer. Vir gene action generates and processes a T-DNA copy and facilitates T-DNA movement out of the bacterium and into the plant cell. Helper plasmids for non-oncogenic plant transformation have been developed to utilize the vir gene functions with T-DNAs containing genes of choice (Hood *et al.* 1993).

The removal of the oncogenes from the Ti plasmid results in disarmed strains of *A. tumefaciens* (Klee *et al*. 1987). The oncogenes of *Agrobacterium* are replaced by reporter genes / screenable marker genes (e.g. β-glucuronidase gene (gus), luciferase (luc) gene for analyzing gene expression. Genes conferring resistance to antibiotics (e.g. neomycin phosphotransferase II (nptII), hygromycin phosphotransferase (hpt), phosphinothricin acetyl transferase (bar) are used to allow selection between transgenic and non transgenic cells. Also oncogenes have been replaced by genes of economic importance (McElroy & Brettel 1994).

Plants are usually transformed with relatively simple constructs, in which the gene of interest is coupled to a promoter of plant, viral or bacterial origin. Some promoters confer constitutive expression while others may be selected to permit tissue specific expression. The cauliflower mosaic virus (CaMV) 35S RNA promoter is often used because it directs high levels of expression in most plant tissues (Walden & Wingender 1995).

5.1.2.1. Mechanism of *Agrobacterium* infection, T-DNA transfer and integration

Plant species differ greatly in their susceptibility to infection by *Agrobacterium tumefaciens* or *rhizogenes*. Even within a species, different cultivars or ecotypes may show different degrees of susceptibility. These differences have been noted in a variety of plant species. The subject matter has been reviewed (Gelvin 2000). Though environmental or physiological factors are attributed for these differences, genetic basis for susceptibility has recently been described in *Arabidopsis* (Nam *et al.* 1997).

Agrobacterium attaches to plant cells in a polar manner in a two-step process. The first step is likely mediated by a cell-associated acetylated, acidic capsular polysaccharide (Reuhs *et al.* 1997). The second step involves the elaboration of cellulose fibrils by the bacterium, which enmeshes large numbers of bacteria at the wound surface (Matthysse *et al.*1995).

The interaction between *Agrobacterum* spp. and plant involves a complex series of chemical signals communicated between the pathogen and the host cells. These signals include neutral and acidic sugars, phenolic compounds, opines (crown gall specific molecules synthesized by transformed plants), Vir (virulence) proteins and the T-DNA (Gelvin 2000). Baker *et al*. (1997) has described the chemical signaling in plant-microbe interactions. The T-DNA transfer process initiates when *Agrobacterium* perceives certain phenolic compounds from wounded plant cells (Hooykass & Beijersbergen 1994) which serves as inducers or co-inducers of the bacterial *vir* genes. Phenolic chemicals such as acetosyringone and related compounds (Dye *et al*. 1997) are perceived via the VirA sensory proteins (Doty *et al*.1996). Most of the induced Vir proteins are directly involved in T-DNA processing from the Ti plasmid and the subsequent transfer of T-DNA from the bacterium to plant. Among them VirD2 and VirE2 contain plant active nuclear localization signal sequences (NLS) (Herrera-Estrella *et al*. 1990). VirD2 protein is directly involved in processing the T-DNA from the Ti plasmid. It nicks the Ti plasmid at 25-bp directly repeated sequences, called T-DNA borders that flank the T-DNA (Veluthambi *et al*. 1988). Thereafter, it strongly associates with 5' end of the resulting DNA molecule (Filichkin & Gelvin 1993) through tyrosin (Vogel & Das 1992). VirD2 contains two nuclear localization signal (NLS) sequences (Herrera-estrella *et al*. 1990) whereas VirE2 contains two separate bipartite nuclear localization signal (NLS) regions that can target linked reporter proteins to plant cell nuclei (Citovsky *et al.*1994).

Many plant species are still recalcitrant to *Agrobacterium* transformation. This recalcitrance does not result from a lack of T-DNA transfer or nuclear targeting, rather its integration into the genome of regenerable cells appears to be limiting. In the future, it may be possible to overexpress endogenous genes involved in the integration process or to introduce homologous genes form other species, and thereby effect higher rates of stable transformation (Gelvin 2000).

5.1.3. *Agrobacterium tumefaciens* (At) mediated transformation studies in cotton

Reports on *Agrobacterium*-mediated gene transfer in cotton have been listed earlier (Table 1.12, Chapter 1). Umbeck *et al*. (1987) have described transformation of Coker varieties 310, 312 & 5110 of cotton using hypocotyl sections inoculated with *Agrobacterium* strain LBA 4404 containing plasmid CMC 1204. The plasmid contained a gene for neomycin phosphotransferase (NPT II) and chloramphenicol acetyltransferase (CAT). Three transgenic plants were identified that expressed NPTII and CAT. Southern analysis indicated that portions of the modified T-DNA region were deleted in one regenerant.

In an another report in the same year, Firoozabady *et al*. (1987) used *Agrobacterium* strain LBA 4404 for transformation of cotyledon pieces from Coker 201. The plasmid pH575 contained NPTII and octopine synthase (OCS) genes. Both the genes expressed at varying levels in transgenic plants. Southern analysis confirmed the presence of NPTII in all 15 plants, however, two plants were found to be octopine negative. The whole process from infection to transfer of transgenic plants to soil took 6-8 months.

Perlak *et al*. (1990) developed transgenic plants of cotton cultivar Coker 312 via *Agrobacterium tumefacines* strain A208 containing cointegrates of a disarmed nopaline plasmid and the intermediate vectors pMON5377 or pMON5388. The plasmids harbored insecticidal genes of *Bacillus thuriengiensis* var. *kurstaki* HD-1 (*cryIA (b*) and HD-73 (*cryIA (c)* driven by CaMV 35S promoter. They used the transformation and regeneration protocols as described by Umbeck *et al*. (1987); Firoozabady *et al*. (1987) and Trolinder & Goodin (1987). Transgenic plants assayed under high insect pressure with *Heliothis zea* (cotton bollworm) showed effective square and boll protection.

Bayely *et al*. (1992) have engineered the 2,4-D resistance trait in cotton cultivar Coker 312 by transferring the 2,4-D monooxygenase gene *tdf*A from *Alcaligenes eutrophus* via *Agrobacterium*-mediated transformation. Vector pBIN19::pR07 containing the *tfd*A gene was

initially transferred into the disarmed *Agrobacterium tumefaciens* strain GV3111 harboring the helper Ti plasmid pTiB6S3S3. Cotton plants obtained were tolerant to three times the field level of 2,4-D used for wheat, corn, sorghum and pasture crops.

Herbicide resistant transgenic cotton plants carrying a mutant forms of a native acetohydroxyacid synthase (AHAS) have been developed by Agrobacterium-mediated transformation (Rajasekaran *et al*.1996 b). The mutant forms of A19 gene were subcloned into polylinker of binary vector Bin19 and were introduced into *A.t* strain A136/542. The cotyledons and hypocotyl explants were used for *A.t* transformation and procedures of transformation were followed as described by Firoozabady *et al.* (1987).

The expression of Protease inhibitor gene in cotton cultivar Coker 312 has also been reported via *A.t*-mediated gene transfer (Thomas *et al*. 1995). Cotton transformation and regeneration procedures followed in this case were largely as described by Firoozabady *et al*. (1987) and Bayley *et al.* (1992).

Although cotton has been transformed by *Agrobacterium* mediated methods and plants have been subsequently regenerated (as mentioned above), commercially important cultivars have proven very difficult to regenerate. Consequently, *Agrobacterium*-mediated transformation of cotton has been limited to those specific cultivars that can be regenerated in tissue cultures. To overcome this difficulty, Zapata *et al.* (1999 a) used shoot apex as an explant for *Agrobacterium* mediated transformation in cotton. They transformed Texas cultivar CUBQHRPIS using *A.t* strain LBA 4404 containing plasmid pBI121. The vector contained a β-glucuronidase (GUS) gene driven by CaMV 35S promoter and neomycin phosphotransferase (NPTII) gene driven by Nos promoter. Out of a total 1010 *Agrobacterium* treated shoot apices, eight plants grew on kanamycin selection and were transferred to soil. Progeny obtained by selfing were germinated in the green house. Evidence for integration of the GUS gene was observed in two successive generations from the regenerants.

5.1.4. Micrografting

Micrografting consists of grafting of a shoot apex from a mother plant or a microshoot onto a young plant grown in the greenhouse / nursery or onto a decapitated seedling under aseptic growth conditions (Jonard 1986). Micrografting has been successfully applied in the tissue culture regenerants in citrus (Pasquale *et al.* 1999) and cotton (Luo & Gould 1999; Banerjee *et al.* 2000) and recovery of putative transformants in a grain legume (Pickardt *et al.*

1995; Krishnamurthy *et al*. 2000). It has been reported that in general, putative transformants are difficult or fail to root (Pickardt *et al*. 1995), as they are subjected to high concentrations of antibiotics for prolonged periods during selection.

Though cotton has been transformed and transgenic plants have been developed, the success is mostly restricted to American cotton especially Coker varieties. Till date, reports on transformation of Indian cotton cultivars is not available in literature. Also until recently, success on regeneration of Indian cultivars was not reported. Ours is the first report on regeneration of Indian cultivars of cotton. Hence, we undertook the work on genetic transformation of Indian cultivars with the following objectives:

1. Optimization of cocultivation with *Agrobacterium* to achieve transformation in cotton cultivars through embryo axis explants.
2. Histochemical and Southern analysis of the transformants,
3. Optimization of an *in vitro* micrografting procedure for recovery of putative transformants.

5.2. Materials and methods

5.2.1. Plant material

Delinted seeds of Indian cultivar of cotton NHH-44, LRK-516, LRA-5166, DHY-286 and DCH-32) all *Gossypium hirsutum* L, varieties were surface sterilized and germinated aseptically as described in chapter 2. Embryo axes were excised from seeds and their radicles were discarded leaving 2 mm long structures (embryo axes) which were used as explants. These 2 mm long embryo axis were split longitudinally (Fig. 5.1A, B) to expose the meristematic region

5.2.2. *Agrobacterium* strain

The *Agrobacterium tumefaciens* strain pGV2260 was used for the present study. The strain contains the plasmid p35SGUSINT which is a pBin19 derivative, carrying a chimeric NPTII gene and a GUS gene construct with a ST-LS1 gene derived intron under the control of CaMV 35S promoter (Vancanneyt *et al.*1990) (Fig. 5.2 A, B). The strain was supplied by Prof. Deepak Pental (University of Delhi, North Campus, New Delhi, India).

5.2.3. Bacteriological media

E.coli strain (pHP23) and *Agrobacterium* strain (pGV2260) were grown in LB (Sambrook *et al.* 1989) and YEB medium (Shaw 1988) respectively. The pH of LB and YEB media was adjusted to 7.0 and 7.2 respectively. Compositions of both the media are given in Table 5.1.

Table 5.1: Compositions of LB and YEB media.

Compositions	LB medium (gm / l)	YEB medium (gm / l)
Bacto-tryptone	10	-
Bacto-yeast extract	5	1
NaCl	10	-
Beef extract	-	5
Bacto-peptone	-	5
Sucrose	-	5

$MgSO_4$	-	0.5

LB – (Luria-Bertani Medium); YEB - (Yeast extract medium)

Fig. 5.1

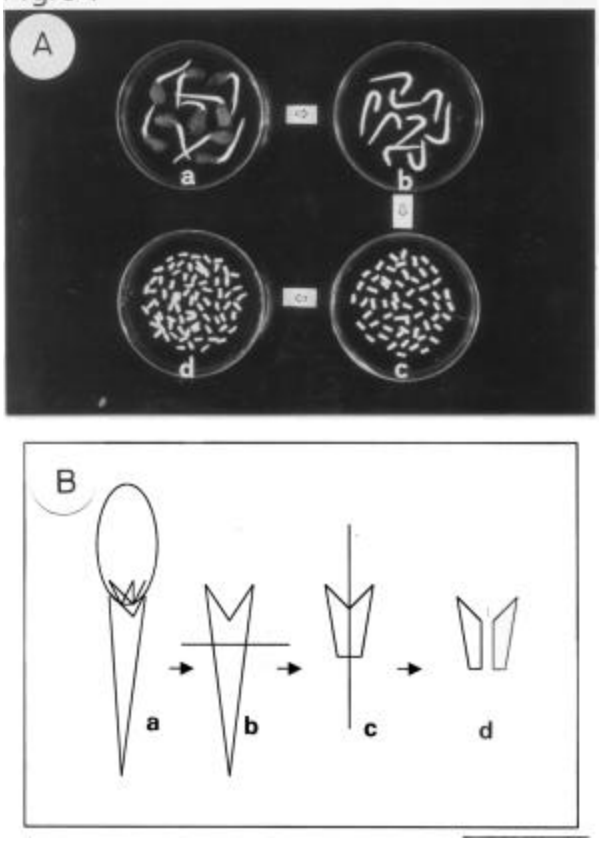


Fig. 5.1

A. Preparation of embryo axes explants for *Agrobacterium* treatment: a=germinated seeds, b=embryo axes, c= embryo axes without radicle, d=split embryo axes.

B. Diagrammatic representation of embryo axis explant preparation: a=germinated seed, b=embryo axis, c=embryo axis without radicle, d= split embryo axis.

Fig.5 2

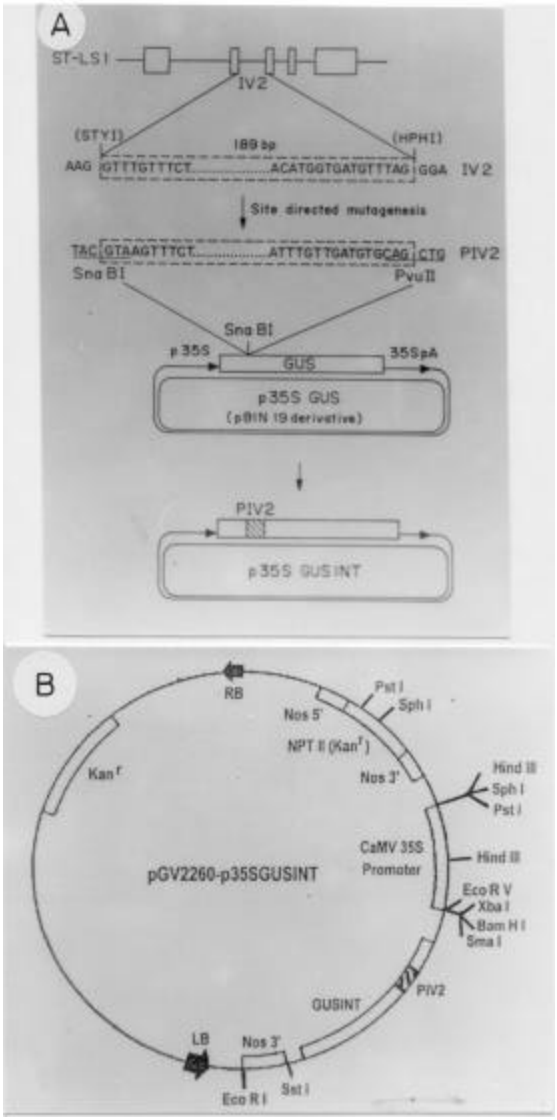


Fig. 5.2

A. *Agrobacterium* map of pGV2260-p35SGUSINT used in the present study. Source: Vancanneyt *et al.* (1990). The ST-LS1 gene derived portable intron (IV2) is cloned in the β-glucuronidase (GUS) gene. The drawing also shows p35SGUS and its intron derivative p35SGUSINT.

B. Plasmid map of *Agrobacterium* strain pGV2260.

5.2.4. Growth media and conditions for *Agrobacterium*

The *Agrobacterium* strain was grown in YEB medium supplemented with antibiotics such as Kanamycin (100 mg/l), Streptomycin (50 mg/l) and Rifampicin (100 mg/l). Culture was grown at 200 rpm on a rotary shaker at 28 °C for 16 h. Aliquots of *Agrobacterium* culture at hourly intervals were pipetted out and the optical density of the culture was measured at 600nm. The optical density was plotted against time to give the characteristic growth curve (Fig. 5.3). The culture after 18 hour starts showing a decline in the optical density and thereby 18 hour grown culture was used for all the experiments.

5.2.5. Regeneration media

Explants were cultured either in a medium containing Murashige and Skoog's (MS) salts+Gamborg's (B5) vitamins without phytohormones (herein after referred as MS0) or (a) containing Murashige and Skoog's (MS) salts+Gamborg's (B5) vitamins supplemented with BAP (0.1) + NAA (0.02mg/l) and Sucrose 2% (hereinafter referred as MS1) or in a medium (b) containing MS salts+B5 vitamins supplemented with TDZ (0.1) + NAA (0.01mg/l) + glucose 3% (hereinafter referred as MS2). Both the media were solidified with 0.65% agar (Hi-media, India).

5.2.5.1. Determination of lethal dose of kanamycin

The lethal dosage (LD_{50}) for kanamycin was determined by inoculating embryo axis explants on medium MS1 and supplemented with various concentrations of kanamycin (25, 50, 75, 100, 150 mg/l). The cultures were incubated for four weeks at 30 °C under a 16 h photoperiod at light intenstiy of 27 $\mu Em^{-2}s^{-1}$ provided by cool white fluorescent tubes.

5.2.5.2. Cocultivation of explants

Longitudianlly split embryo axis explants were treated with *Agrobacterium tumefaciens* strain GV2260. *Agrobacterium* culture (18 h grown) was spun at 5000 rpm for

10 min. at ambient temperature. The pellet obtained was resuspended in 3 ml of liquid medium of MS1 (suspension S1) or in 200 µl of liquid medium of MS2 (suspension S2). Treatment of the explants was carried out by two methods: dipping of embryo axes in a batch of 40 with *Agrobacterium* suspension (S1) for 3-4 sec, 5, 10, 20 and 30 min or spotting of 2 µl of *Agrobacterium* suspension (S2) on the individual split embryo axis. In this manner, around 500 explants per experiment were treated. In a separate set of experiment, 150 explants each for dipping and spotting treatments were taken.

Fig. 5.3 A

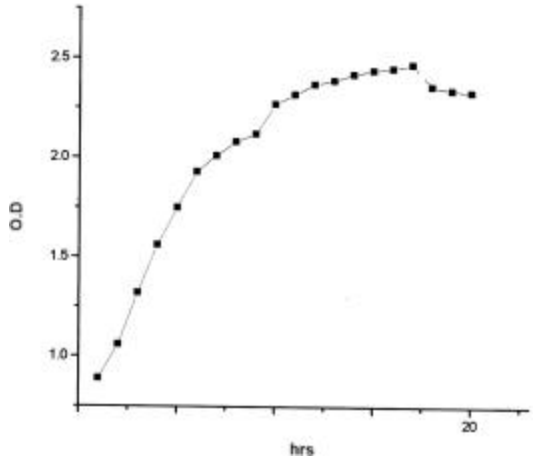


Fig. 5.3

A. Characteristic growth curve of *Agrobacterium* strain.

All the explants treated by dipping were blotted dry and co-cultivated on semisolid medium MS1 for 72 h. These were then washed thrice with sterile double distilled water containing 500 mg/l of Claforan (Russel India Ltd), blotted dry again and cultured on semi-solid MS1 medium containing kanamycin (50 mg/l) + Claforan (500 mg/l). Explants not treated with *Agrobacterium* and cultured on medium containing kanamycin (50 mg/l) served as controls. Cultures were incubated under 16 h photoperiod (light intensity of 27 $\mu Em^{-2}s^{-1}$) at 30°C for 4 weeks.

Explants treated by spotting were co-cultivated at 30 ^{0}C at a light intensity of 27 $\mu Em^{-2}s^{-1}$ for 72 hrs. After that, these were washed as above and cultured on MS2 medium containing kanamycin (50 mg/l) and claforon 500 mg/l). These were incubated for 4 weeks as mentioned above.

5.2.5.3. Development of shoots

Shoots developed on MS1 medium containing kanamycin (50 mg/l) and claforan (500mg/l) after 3-4 weeks of incubation were transferred to a freshly prepared medium and were incubated for another 3-4 weeks. The shoots surviving this selection pressure were again transferred to MS1 medium with half the concentration of kanamycin 25 mg/l and claforan (250 mg/l) and were incubated for further 4 weeks. The shoots surviving on selection pressure for 3 months were grafted onto decapitated rootstocks (details given in section 5.2.7).

5.2.5.4. Development of callus from embryo axis

In separate sets, calli developed from split embryo axes on MS2 medium containing kanamycin (50 mg/l) and claforan (500 mg/l) were transferred to freshly prepared medium. After six weeks of incubation, calli were again transferred to fresh medium having kanamycin (50mg/l) and half concentration of claforan (250 mg/l). The calli growing on the selection pressure were tested for GUS assay at different period of intervals.

5.2.6. GUS assay

Histochemical analysis was carried out to determine the β-glucoronidase activity in embryo axis and cotyledonary leaf after three weeks of treatment of *Agrobacterium temefaciens*. However, the leaves of the putative transformants were tested after two months of survival in selection pressure. Tissues were cut into small pieces and immersed in 1mM X-Gluc solution 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. Details of the GUS solution are given in Table 5.2.

Table 5.2: Reagent mix for GUS assay.

Stock solution	Final concentration	Reagent Mix ml/ml
1.0 M NaPO4 buffer, pH 7.0	0.1M	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
Subtotal		400
Distilled water		600
Final volume		1000

5.2.7. *In vitro* micrografting of putatively transformed cotton shoots

In vitro micrografting was carried out with nine putatively transformed shoots of cotton. Ten days old seedlings of cotton cultivar DCH-32 grown under sterile conditions and decapitated about 3 cm above the root: shoot joint served as the rootstocks. A longitudinal slit of about 1 cm deep was made in the center of the cut end of each rootstock. These were placed vertically in Laxenta box containing 80 ml of semi-solid medium of MS salts+B5 vitamins, 2 % sucrose and 0.65 % agar. Tiny putatively transformed shoots (about 2-3 cm long) were taken out of the selection medium and their bases were obliquely cut from both the sides. These were inserted into slits made in the rootstocks. All the grafts were incubated at 25 °C under16 h photoperiod of light intensity of 16 $\mu E\ m^{-2}\ s^{-1}$. Observations were taken after one months of incubation.

Since the number of putatively transformed shoots was limited, micrografting experiments were carried out with control shoots to optimize the procedure. The optimized method was then utilized for micrografting of nine putative transformants. Attempts were also made with control shoots to test the feasibility of direct micrograftng under *ex vitro* conditions.

5.2.8. *In vitro* and *ex vitro* micrografting of control shoots

5.2.8.1. Preparation of scion

Seeds of cotton cultivar NHH-44, DCH-32 (*Gossypium hirsutum* L.), and AKH-081 (*Gossypium arboreum*) were surface sterilized, disinfected and germinated as mentioned in chapter 2. Embryo axes (2 mm) from the germinated seeds of cotton cultivar NHH-44 were dissected out discarding radicles as described before. These were cultured on a medium containing MS salts (Murashige & Skoog 1962) + Gamborg's vitamins (B5) (Gamborg *et al*. 1968) + 3 % glucose + 0.21 % gelrite in 85 mm plastic petri dishes. The pH of the medium was adjusted to 5.8 before autoclaving. Explants were incubated at 30 °C under 16 h photoperiod of light intensity of 27 $\mu E\ m^{-2}\ s^{-1}$. Each petri dish contained 20 explants. Tiny shoots (about 0.6-

0.7 cm long) that developed from the embryo axes were used as scions. Their bases were obliquely cut from both the sides before grafting them onto rootstocks.

5.2.8.2. Preparation of rootstock

Surface sterilized and disinfected seeds of cotton cultivars DCH-32 and AKH-081 after emergence of radicles were transferred to test tubes with MS salts and vitamins, 2 % sucrose and 0.65 % agar. The germinated seeds of DCH-32 were also transferred to earthen pots containing a mixture of sand: soil: compost (1:1:1). The culture tubes were kept for 10 days in darkness (for etiolation) or in light conditions (non-etiolation), while earthen pots were kept at 25 °C under 16 h photoperiod of light intensity of 16 $\mu E\ m^{-2}\ s^{-1}$. *In vitro* grown 10 days old seedlings and seedlings grown in earthen pots were used as rootstocks for *in vitro* and *ex vitro* grafting experiments respectively.

5.2.8.3. *In vitro* micrografting

In vitro micrografting was carried out in polypropylene Laxenta boxes. Ten days old seedlings of DCH-32 (both etiolated and non-etiolated) grown under sterile conditions and decapitated about 3 cm above the root: shoot joint served as the rootstocks. A longitudinal slit of about 1 cm deep was made in the center of the cut end of each rootstocks and these were placed vertically in Laxenta box containing 80 ml of liquid or semi-solid medium of MS salts, B5 vitamins, 2 % sucrose and 0.65 % agar. The rootstocks were supported in vertical position in liquid medium with the help of perforated filter paper. The scions were inserted into slits of the rootstocks. Twenty grafts under *in vitro* conditions were made for each set of experiment.

5.2.8.4. Ex-vitro micrografting

Ex-vitro micrografting was carried out in earthen pots. For *ex vitro* micro grafting, seedlings raised in earthen pots were decapitated about 3 cm above the soil and about 1 cm deep slit was made. Microshoots were inserted on the rootstocks and the pots were immediately enclosed in transparent polybags. The grafting joint was not tied or covered in the present experiment. Thirty grafts were made under *ex vitro* conditions.

5.2.8.5. Culture conditions

All the grafts were incubated at 25 °C under 16 h photoperiod of light intensity of 16 $\mu E\ m^{-2}\ s^{-1}$. Observations on growth parameters (scion length, fresh weight and number of nodes per plant) were recorded after 30 days of incubation.

5.2.8.6. Histology of the graft union

About 1 cm long segment of the graft-union of a 25 days old graft was excised and fixed in formalin: acetic acid: ethanol (5:5:90) for 48 h. Histology of the graft union was carried out as described in chapter 2.

5.2.8.7. Hardening of the grafted plants

Grafted plants were carefully removed after 30 days from the Laxenta boxes; the roots were washed thoroughly to remove the adhering medium. Plants were transferred to earthen pots containing a mixture of autoclaved soil: sand: compost (1:1:1) and were immediately covered with transparent polybags. The pots were kept at 25 °C under 16 h photoperiod of a high light intensity of 47 $\mu E\ m^{-2}\ s^{-1}$. These grafted plants were sprayed with 1 % fungicide solution (Bavistin, BASF India Ltd.,) and irrigated with sterile water every 3rd day and were supplied with the nutrient solution (1/10th concentration of MS major salts + full minor salts) twice a week for 30 days. The plants were acclimatized by trimming corners of polybags. Thereafter these pots were kept in the green house for 4 weeks and were later transplanted to the field.

5.2.9. Plant Genomic DNA isolation

Genomic DNA (Fig. 5.4A) was isolated by the protocol routinely followed in our laboratory is described below:

1. Crush 1 gm of leaf or callus tissue in liquid nitrogen in a pre-cooled mortar and pestle to a fine homogeneous powder.

↓

2. Add (3 ml of buffer A + 1% PVP) and 3 ml of buffer B individually to the frozen powder and mix the mixture with the pestle gently

↓

3. Distribute the mixture (0.6 ml each) with the help of a wide bore pipette tip to 2 ml capacity eppendorf tubes

↓

4. keep in a water bath at 65°C for 10 min.

↓

5. Add equal volume (0.6 ml) of CH: IAA (24:1) to the mixture and vortex gently for few seconds

↓

6. Centrifuge at 10000 rpm for 10 min. at RT

↓

7.Collect upper phase and repeat steps 5 and 6 two times

↓

8. Add double the volume of 96% chilled ethanol to the aqueous phase and mix gently by inverting the capped tubes 10-15 times and keep at – 20 ° C for one hour.

↓

9.Centrifuge at 12000 rpm for 10 min at 4 ° C

↓

10. Remove the supernatant and invert the tube on a paper towel to dry the pellets

↓

11. Add 100 µl of MQ water to the pellet and swirl the tube gently to dissolve the pellet, if the pellet is not dissolved, then keep at 65°C in water bath for 10 - 20 min.

↓

12. Add equal volume of 2X concentration of 1M NaCl + 0.1M Tris + 1mM EDTA

↓

13. Mix gently and add RNAse at a concentration of 10µg/ml

↓

14. Keep at 37 ° C for 2-3 hrs.

↓

15.Add equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1)

↓

16.Mix by mild vortexing for 1-2 sec. and centrifuge at 10000 rpm for 5 min. at RT

↓

17.Collect upper phase and add equal volume of CH: IAA

↓

18.Repeat steps 15,16 and 17 two to three times

↓

19.Collect upper phase and add 3 volumes of ice cold 96% ethanol

↓

20. Mix gently and keep at – 20 ° C for 1 h or – 70 ° C for 30 min.

↓

21. Centrifuge at 10000 rpm for 15 min at 4 ° C

↓

22. Remove the supernatant and invert the tube on a paper towel to dry the DNA

↓

23. Add 60 µl of sterile Milli Q water and dissolve the pellet

↓

24. Add chilled 2 volume of 7.5 M Ammonium acetate and mix gently

↓

25. Add 3 volume of chilled ethanol and mix gently by inverting the tube

↓

26. Keep at – 20°C for 1 hr

↓

27. Centrifuge at 10000 for 15 min at 4°C

↓

28. Remove the supernatant completely by inverting the tube on a paper towel

↓

29. Add 1 ml of 70 % ethanol to the pellet and swirl the tube

↓

30. Centrifuge at 10000 for 15 min at 4°C.

↓

31. Remove the supernatant and dry the pellet by inverting the tube on paper towel

↓

32. Dissolve the pellet in sterile Milli Q water.

Buffer A = 2 x CTAB solution; buffer B = 8M Lithium Chloride solution; PCA = Phenol: Chloroform: Isoamylalcohol; CH: IAA = Chloroform: Isoamyl alcohol; PVP = Polyvinylpyrolidone (PVP40).

5.2.8. Plasmid DNA isolation

Plasmid DNA was isolated from the strain pHP23 by standard alkaline lysis method (Sambrook *et al*. 1989). Isolated DNA was given RNAse treatment and purified with phenol: chloroform method.

5.2.10. Preparation of probe for hybridization

The plasmid DNA isolated from pHP23 was digested with *Hind*III (Boehringer Mannheim, Germany). The 0.85-kb NPTII fragment was isolated and purified by Qiaex II agarose gel extraction method (QUIAGEN Gel Extraction Kit, Germany). The fragment was labeled with $^{\alpha 32}$p by standard random prime labelling method. For non- radioactive Southern, the NPTII fragment was also labeled with digoxigenin using DIG non radioactive kit (Boehringer Mannheim, Germany).

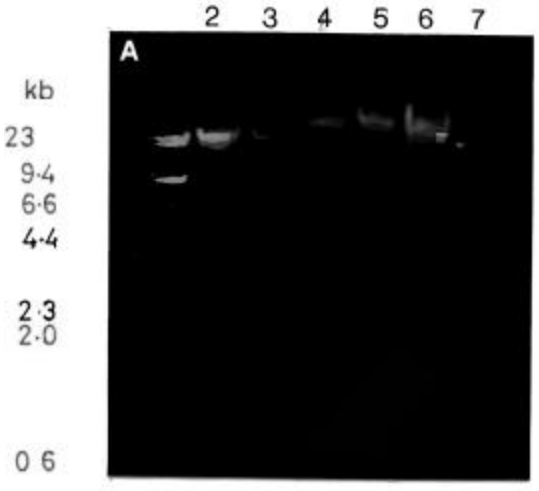
5.2.11. Southern hybridization

Southern hybridization was carried out by non-radioacitve and radioacitve methods to confirm the integration of NPTII gene in cotton callus tissue and putatively transformed shoots. The non-radioacitve Southern was performed by using DIG DNA labeling and DIG Luminescent Detection Kit and the methodology was followed as described in the DIG system user's guide manual (Boehringer Mannheim, Germany).

To carry out radioactive southern, DNA fractions (15 µg) from negative control (Non transformed cotton callus tissue), transformed callus of cotton, putatively transformed plants, and

positive control (transformed tobacco) were digested with *Hind*III (10 units/μg of DNA) for overnight at 37 °C. Digested DNA samples were electrophoresed on a 1% agarose gel for a period of 6 hrs at 50 milli amps. in 1 x TAE buffer. The gel was stained with ethidium bromide (0.5 μg/ml) for 30 min and destained in double distilled water for 15 min. Digested DNA patteren was visualized and photographed (Fig. 5.4B). The gel was blotted onto Hybond positively charged nylon membrane (Boehringer Mannheim, Germany) and DNA was capillary transferred to membrane by standard alkali transfer method where 0.4 M NaOH was used as transfer reagent. DNA was fixed by baking the membrane for 2 h at 80 °C. Prehybridization was carried out for 6 h at 65 °C and then boiled radiolabelled probe was added to the hybridization solution. Hybridization at 65 °C was continued for 24 hrs. The membrane was washed for 15 min at RT in 2 x SSC containing 0.1% SDS. Thereafter the membrane was given 2 washes for 30 min each with 0.5 X SSC containing 0.1% SDS. at 60 ° C. Autoradiography was carried out by exposing the membrane to X-ray film at - 70 ° C for 5 days.

Fig 5.4



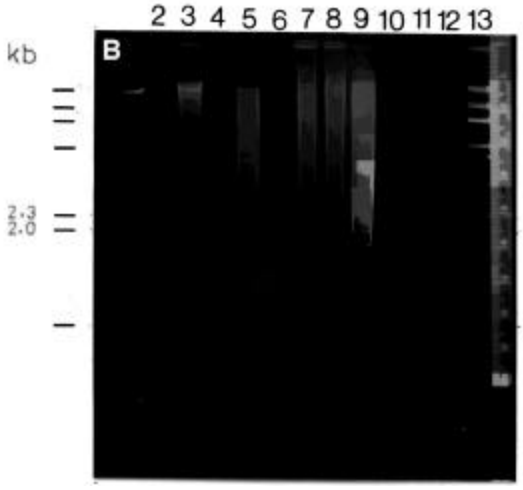


Fig. 5.4

A. Agarose gel electrophoresis of plant genomic DNA isolated from: control callus (lane 2), transformed callus (lane 3), putatively transformed plants of cultivar NHH-44 (lane 4 & 5) and transformed tobacco (lane 6). Lanes 1 & 7 are λ- DNA *Hind*III digest molecular weight maker.

B. Agarose gel electrophoresis of *Hind*III digested plant genomic DNA from: control callus (lane 3), transformed callus (lane 5), putatively transformed plants of cultivar NHH-44 (lane 7 & 8) and transformed tobacco (lane 9). Lanes 1 & 13 are λ- DNA *Hind*III digest molecular weight maker. Lane 11 is the 0.85 kb fragment of NPTII from pHP23.

5.3. Results and discussion

Though cotton has been transformed by *Agrobacterium* and plants have been regenerated (Umbeck *et al*. 1987; Firoozabady *et al.* 1987; Perlak *et al.*, 1990), success has been limited to Coker varieties that are highly regenerable. Commercially important cultivars have proven very difficult to regenerate under culture conditions.

In the present study, an attempt has been made to develop a method for transformation of embryo axis explants of cotton cultivars. Explants were cocultivated with *A. tumefaciens* strain containing GUS gene interrupted by an intron to avoid false positives. The selection pressure was applied immediately after the co-cultivation period.

It is now widely accepted that the most suitable explants for transformation are those that require the least amount of time in tissue culture before and after the transformation step. Preferred explants for transformation includes: immature embryos, proliferative shoot cultures and embryo axis derived from mature or immature seed for direct meristem transformation in dicotyledons (Christou 1996). In our study, *Agrobacterium*-mediated transformation in embryo axis explants is described below:

5.3.1. Lethal dosages of kanamycin

Elongation of embryo axes was observed on a medium supplemented with or without kanamycin (25-150 mg/l). Explants on medium without kanamycin (controls) developed normal shoots after 3 weeks of incubation. Embryo axes cultured on medium with kanamycin 25 and 50 mg/l showed initial shoot development, however, these could not grow further and bleached. Survival of a few shoots was observed on kanamycin 25 mg/l, however, concentrations 50 mg/l and higher were lethal and inhibited the shoot development completely (Fig. 5.5A). Hence, kanamycin at 50 mg/l was taken as the selection pressure in all the experiments.

5.3.2. Cocultivation

In the present section, work after cocultivation has been restricted to cultivar NHH-44 since initial experiments with cultivars LRK-516, DHY-286 and DCH-32 did not yield any shoots surviving on kanamycin (50 mg/l) after 20 min treatment with *Agrobacterium*. GUS activity in any of the tested explants could not be detected (data not shown). A few shoots survived on selection medium after three weeks of culture only in cultivar NHH-44 after *Agrobacterium* treatment. Hence, further experiments were carried out with this cultivar to

optimize the duration and mode of treatment of explants (3-4 seconds, 5, 10, 20, 30, spotting etc.) with *Agrobacterium* suspension to achieve high rates of infection. Treatment of explants for a period of 30 min was not favorable as it posed difficulty in elimination of bacteria from the explants in culture. Cousins *et al*. (1991) have earlier reported that frequency of transformation in cotton is largely influenced by the strain at use, method and period of cocultivation and culture condition.

At the end of the cocultivation period, explants are transferred to a medium containing an antibiotic (cefotaxime in the present study) that will specifically inhibit bacterial growth and a selective agent (kanamycin in the present study) that will select those cells receiving and expressing the gene transfer cassette (Hinchee *et al.* 1994).

Fig 5.5

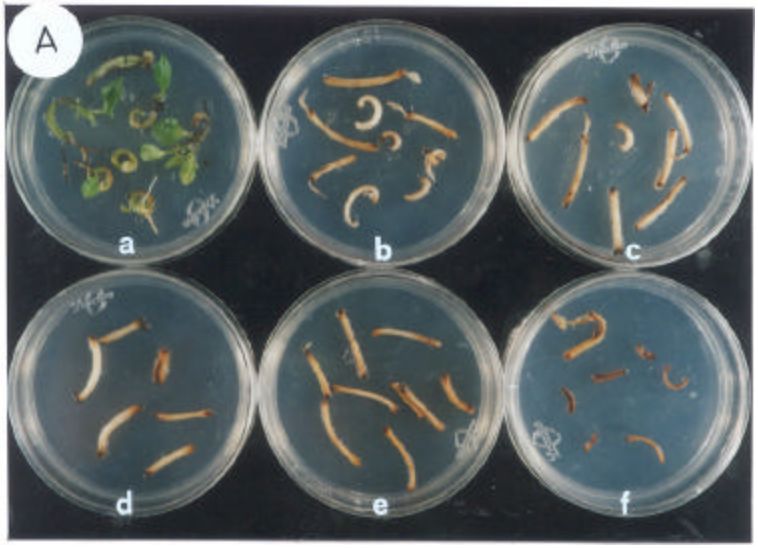


Fig. 5.5

A. Determination of lethal dose (LD50) of kanamycin in cotton embryo axes explants: a= control, b=25 mg/l, c=50 mg/l, d=75 mg/l, e=100 mg/l, f=150 mg/l.

5.3.3. Development of shoots

Though a varied number of explants survived and developed shoots on medium containing kanamycin (50 mg/l) depending on the mode of treatment and after 3 weeks of culture, however their number decreased in subsequent transfer to fresh medium. Treatment of explants for 10, 5 min and 3-4 seconds resulted in a survival of 3, 2 and 2 shoots respectively after 12 weeks of incubation. Also spotting of *Agrobacterium* suspension (S2) on apical regions of embryo axes resulted in survival of 2 shoots (Table 5.3). Altogether 9 shoots survived (putatively transformed) upto a period of three months on selection pressure (Table 5.3). Untreated explants cultured on medium containing kanamycin (50 mg/l) did not survive.

Prior to our study, split embryo axis as explants have not been used for transformation of cotton. Zapata *et al*. (1999 a) have recently reported the use of shoot apex explants from 3-4 day-old germinated seeds and development of transgenic plants in cotton after 3 months of incubation. Rooting of the transformants was achieved by transferring them to kanamycin free medium. Contrast to this, in our study, shoots surviving on selection pressure were stunted in growth and did not develop roots even after prolonged incubation on a medium containing kanamycin (Fig. 5.6A). Therefore these were micrografted onto *in vitro* grown rootstocks (details have been described in section 5.3.6).

Embryo axis has become the explant of choice for development of transgenic plants via *Agrobacterium* co-cultivation in chickpea (Kar *et al*. 1996; Krihsnamurthy *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 explant takes 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 avoided if callus phase can be bypassed, (4) and regeneration through embryo axis is genotype independent.

5.3.4. Induction of callus

Split embryo axes treated with *Agrobactrium* by spotting method and cultured on medium MS2 containing kanamycin 50 mg/l and cefotaxime 500 mg/l induced pale white callus

at cut ends after three weeks of incubation. Proliferation of callus was achieved on transfer to fresh medium.

5.3.5. Gus assay

In our experiments, control explants which were not cocultivated with *Agrobacterium* did not show any endogenous GUS activity. The maximum percentage (14.28) of split embryo axes showed GUS activity when *Agrobacterium* suspension (S2) was spotted on the apical region of embryo axis of NHH-44 and when cultured on semisolid medium MS2 for 3 weeks (Fig. 5.6B). Treatment of split embryo axes with *Agrobacterium* suspension (S1) for 5, 10 and 20 min. resulted in 0.98, 1.28 and 1.62 % of explants showing GUS expression (Table 5.3). Axes dipped for a 3-4 seconds resulted in 5.88 % of explants showing GUS expression at the cut surface of the explant (Fig. 5.6C) (Table 5.3). Transformed callus (Fig. 5.7A) obtained from embryo axis showed intense blue color compared to nontransformed callus (Fig. 5.7B).

In the present study, leaf samples excised from the shoots (putative transformants) surviving on selection pressure for 12 weeks of culture did not show any GUS activity. In an earlier report by Cousins *et al*. 1991, on a transformed Australian cultivar, not all the NPTII expressing plants showed GUS activity, presumably because of premature termination of the introduced T-DNA during the transfer process.

Fig. 5.6

A. Shoots (putative transformants) surviving on medium supplemented with kanamycin (50 mg/l) (cultivar NHH– 44).

B. GUS expression in embryo axis explant treated by spotting of the *Agrobacterium* suspension (S2) and tested after three weeks of culture.

C. GUS expression in split embryo axis explant treated by dipping in *Agrobacterium* suspension (S1) for 3-4 seconds and tested after three weeks of culture .

Fig. 5.6

A. Shoots (putative transformants) surviving on medium supplemented with kanamycin (50 mg/l) (cultivar NHH– 44).

B. GUS expression in embryo axis explant treated by spotting of the *Agrobacterium* suspension (S2) and tested after three weeks of culture.

C. GUS expression in split embryo axis explant treated by dipping in *Agrobacterium* suspension (S1) for 3-4 seconds and tested after three weeks of culture .

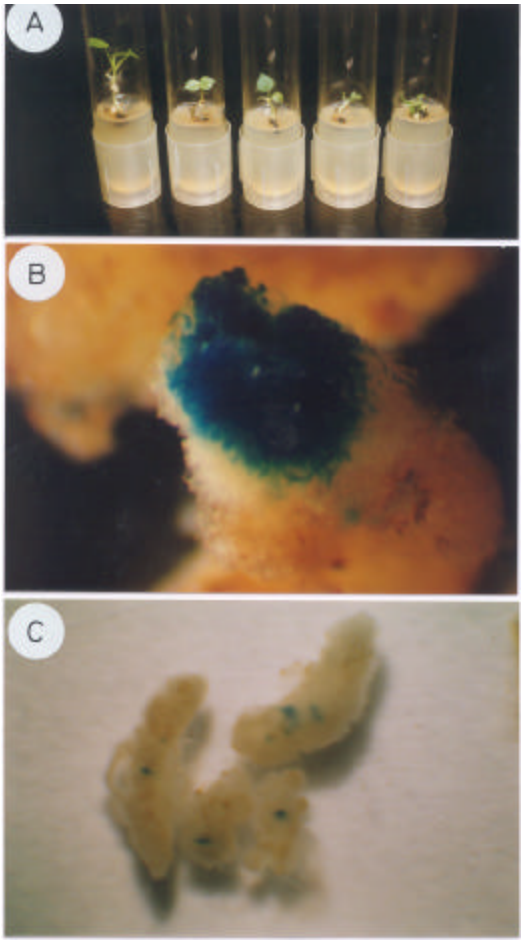

A
B
C

Fig. 5.6

A. Shoots (putative transformants) surviving on medium supplemented with kanamycin (50 mg/l) (cultivar NHH– 44).

B. GUS expression in embryo axis explant treated by spotting of the *Agrobacterium* suspension (S2) and tested after three weeks of culture.

C. GUS expression in split embryo axis explant treated by dipping in *Agrobacterium* suspension (S1) for 3-4 seconds and tested after three weeks of culture .

Fig.5.7

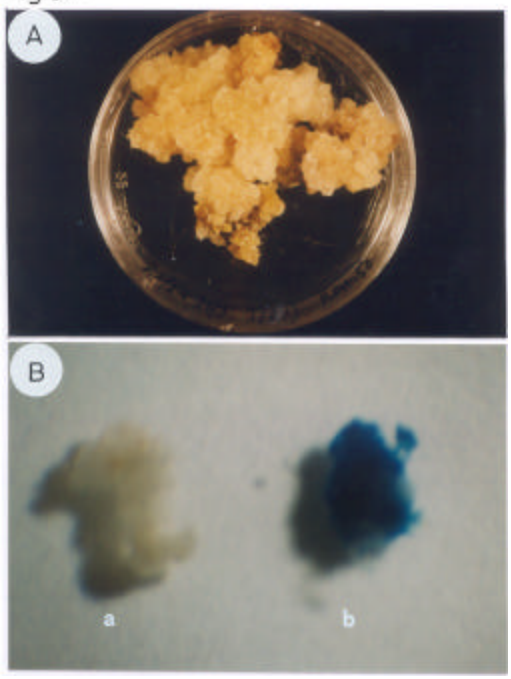


Fig. 5.7

A. Callus derived from transformed embryo axis and maintained on medium supplemented with TDZ (0.1 mg/l), NAA (0.01mg/l) and kanamycin (50 mg/l) (Cultivar NHH-44).

B. Callus a: control (non-transformed), b: transformed (intense GUS expression).

Bacterial β-glucoronidase gene (uidA, 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 & Schell 1990). The assay is extremely sensitive and uses X-gluc (5-bromo-4-chloro-

3-indolyl-β-D-glucuronide), which can qualitatively show cell- and tissue specificity (Jefferson 1989). Cleavage of 5-bromo, 4- chloro, 3-indolyl, β-D-glucoronide (X-gluc) by the enzyme β-glucoronidase produces the final insoluble blue colour precipitate dichlorodibromoindigo (ClBr-indigo) (Fig. 5.8 A). 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 colour, 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 has ruled out the possibility of false GUS activity (blue colour) 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 *Agrobacteria* 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).

Table 5.3: b-glucuronidase expression in split embryo axis of cotton (*G. hirsutum* L) cultivar NHH-44 after inoculation with *Agrobacterium tumefaciens* strain GV2260 / p35SGUSINT.

No. of explants treated	Mode of treatment	Treatment period (min)	No.of explants tested for GUS assays after 3 week of culture	No. of explants found GUS +ve	% of explants showing GUS expression	No. of shoots survived after 3 weeks of culture	No. of putatively trans-formants after 12 weeks of culture
2450	Dipping	30	70	-	0.00	68	0.00
5389	Do	20	123	2	1.62	143	0.00
3245	Do	10	78	1	1.28	129	3.00
2680	Do	5	102	1	0.98	83	2.00
586	Do	3-4 sec.	34	2	5.88	28	2.00
450	Spotting	-	28	4	14.28	21	2.00

5.3.6. *In vitro* micrografting of putatively transformed cotton shoots

Putatively transformed shoots of cotton cultivar NHH-44 (used as scion) were successfully grafted on *in vitro* grown decapitated etiolated seedlings of cultivar DCH-32 (used

as rootstock). Seven out of nine grafted plants survived after hardening (Fig. 5.9A).Grafted plants in pots grew to maturity and developed normal flowers and bolls (Fig. 5.9B). In an earlier report, Pickardt *et al*. (1995) have observed that putative transformants are difficult or fail to root as they are subjected to high concentrations of antibiotics for prolonged periods during selection. In our experiments on transformation with cotton embryo axes, the putative transformants maintained for 12 weeks under antibiotic stress had extremely slow growth and failed to root. This prompted us to attempt micrografting of shoots.

Fig. 5.8 **A**

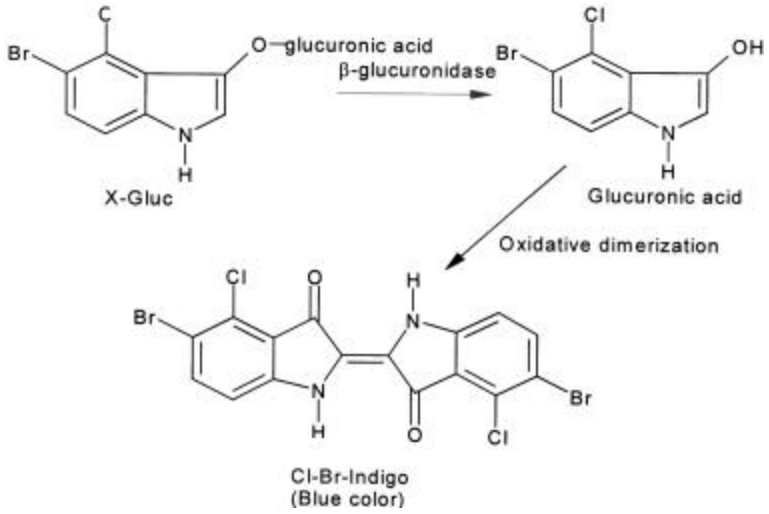


Fig. 5.8

Cleavage of 5-bromo, 4-chloro, 3-indolyl, β-D-glucuronide (X-gluc) by the enzyme β-glucuronidase into dichloro-dibromoindigo (ClBr-indigo).

5.3.7. *In vitro* and *ex vitro* micrografting of control shoots

5.3.7. 1. *In vitro* micrografting

Several aspects such as liquid vs semi-solid medium, rootstocks excised from etiolated vs non etiolated seedlings, intervarietal vs interspecies graft unions, *ex vitro* grafting and subsequent hardening were studied.

Scions which did not wilt and showed growth and elongation of apical part within a few days of culture both in liquid and semi-solid media (Fig. 5.10A) as well as under *ex vitro* conditions were considered to have established graft-unions. Observations made after 4 weeks of culture showed a marked difference in growth parameters in the grafts grown in liquid and semi-solid media (Fig. 5. 10B). The difference was clearly reflected in scion length, fresh weight of grafted plants and the number of nodes per plant (Table 5.4). The maximum elongation of the scion (5.83 cm) and the highest number of nodes (6.3) per plant was observed in grafts made onto the rootstock derived from etiolated seedlings of the cultivar AKH-081 and cultured in liquid medium with a filter paper support. Grafts cultured in liquid medium developed a larger number of roots with greater elongation (Fig. 5.11B) then those on semisolid medium **(**Fig 5.11A).

The maximum number of micrografts (95%) derived from liquid culture and grafted onto the etiolated rootstocks of the cultivar DCH-32 showed survival on hardening. Survival percentage of grafts on semi-solid medium decreased to 65% and 70% for etiolated and non-etiolated (intervarietal) rootstocks respectively. A similar trend was observed when scions were used on etiolated rootstocks (interspecies) of AKH-081 but with higher survival percentages of 90 and 70 for liquid and semi-solid media respectively.

5.3.7.2. Ex vitro micro grafting

Ex vitro grafts had minimum scion elongation (1.41 cm), lowest number of nodes per plant (2.52) and only 30% survival (Table 5.4**).** It has been reported (Hartmann & Kester 1976) that during scion/rootstock union, the new callus tissue that arises from the cambial region

is composed of thin-walled, turgid cells, which easily desiccate and die. Therefore it is important that air moisture around the graft-union is kept at high to promote proliferation of cells in the graft region.

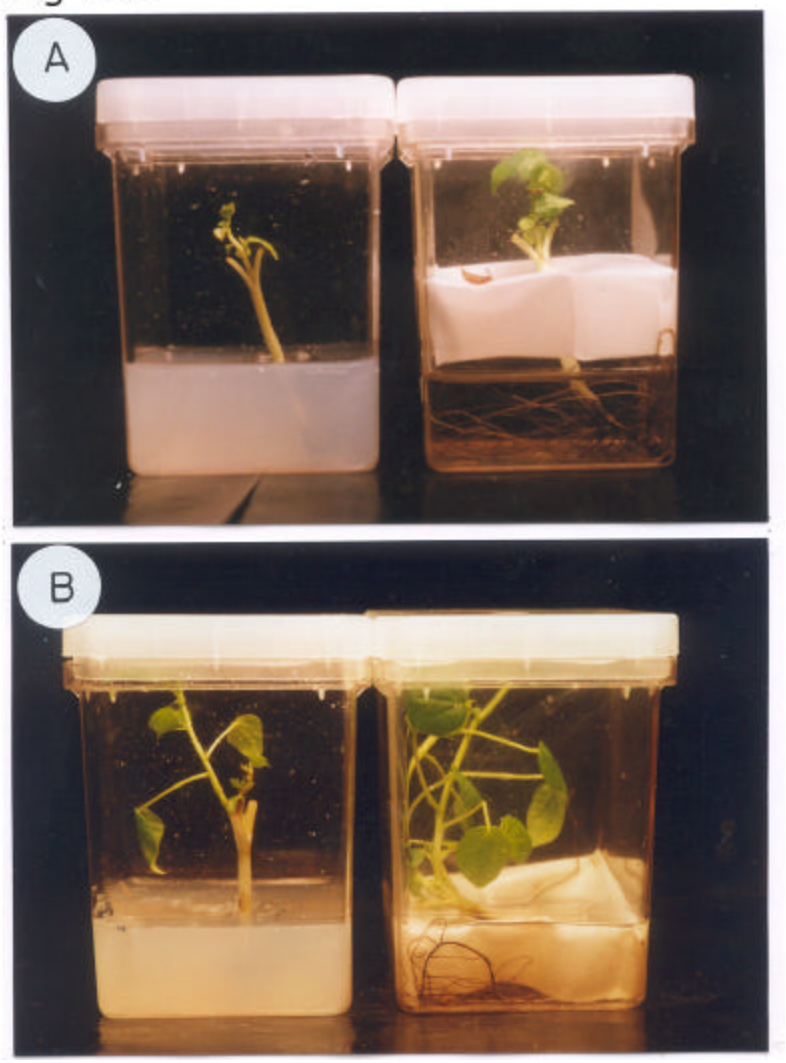
Fig 5.9

Fig. 5.9

A. Putatively transformed grafted plants of cultivar NHH-44 in earthen pots.

B. Putatively transformed grafted plants of cultivar NHH-44 in earthen pots showing flowers (arrow) and boll (arrow) formation.

Fig 5.10



F ig. 5.10

A. Micrografts after two weeks of culture in phytohormone free semi-solid and liquid media.

B. Micrografts after 5 weeks of culture in phytohormone free semi-solid and liquid media.

Fig. 5.11

Fig. 5.11

A. Micrografts taken out from semi-solid medium.

B. Micrografts taken out from liquid medium.

Table 5.4: Effect of culture medium, etiolation and *ex vitro* conditions on growth parameters and the survival of micrografts in cotton.

Conditions	Scion / rootstock	Survival of grafts after hardening		Scion length (cm)	Fresh weight (g)	No. of Nodes / plant
		No.	%			
Liquid Medium / E	Intervarietal	19	95	5.59 ± 1.53	0.78 ± 0.20	5.6 ± 0.95
Liquid Medium /NE	Intervarietal	18	90	5.03 ± 1.01	0.74 ± 0.19	5.1 ± 0.84
Semi-solid Medium / E	Intervarietal	13	65	2.16 ± 0.46	0.36 ± 0.07	5.0 ± 0.83
Semi-solid Medium/ NE	Intervarietal	14	70	2.44 ± 0.75	0.46 ± 0.06	4.72 ± 1.10
Liquid Medium / E	Interspecies	18	90	5.83 ± 1.48	0.71 ± 0.21	6.3 ± 1.50
Semi-solid Medium / E	Interspecies	14	70	2.03 ± 0.81	0.27 ± 0.07	4.18 ± 1.35
Ex vitro grafts / NE	Intervarietal	10	30	1.41 ± 0.36	-	2.52 ± 0.52

E- Rootstocks from etiolated seedlings; NE- Rootstocks from non-etiolated seedlings; ± Standard deviation (each value is mean of 10 replicates each of two experiments; - Data not recorded; growth parameters recorded after 30 days of incubation; differences between the liquid and semi-solid medium conditions were significant at 95% level of confidence.

Inspite of the low success rate, *ex vitro* grafting has some advantages: (1) it is less expensive (2) less time consuming as transplantation from *in vitro* to greenhouse is omitted. Our preliminary results demonstrate the feasibility of *ex vitro* intervarietal grafting in cotton. It should be possible to further enhance the survival percentage of *ex vitro* grafts by manipulating various factors such as the source, size of scion / rootstock and growth conditions.

Histological examination of the graft union showed multiplication of parenchymatous cells by scion and rootstocks (see arrows, Fig. 5.12A) and their integration to form a union. Hardened grafted plants (Fig. 5. 13A) transferred to field grew to maturity and set seed (Fig. 5. 13B, C).

Fig.5.12

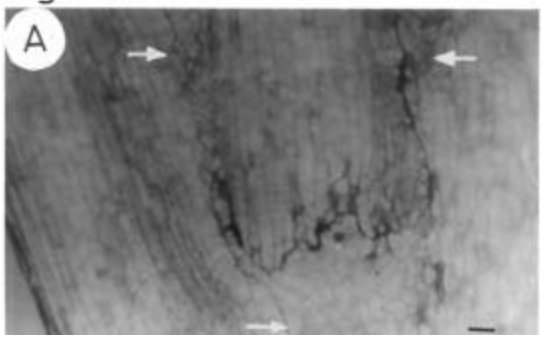


Fig. 5.12

A. Part of longitudinal section of scion-graft union (bar=190 μm).

Fig. 5.13

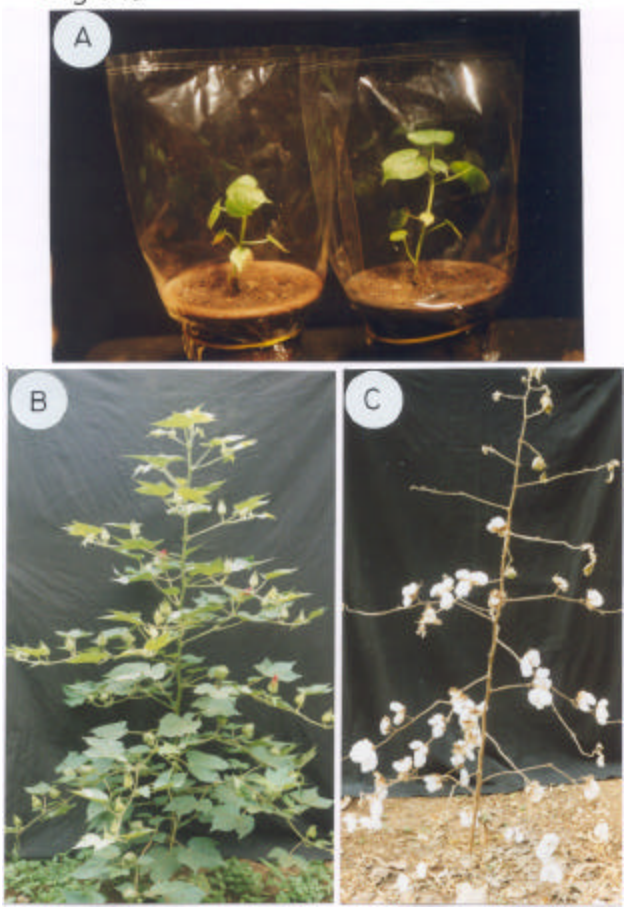


Fig. 5.13

A. Grafted plants covered with polybags for hardening.

B. Control grafted plant of cultivar NHH-44 in field showing flowers and bolls.

C. Dehisced bolls in the grafted plant.

From the above study, it may be concluded that use of etiolated seedlings as rootstock, culturing of micrografts in liquid medium with filter paper supports are the optimum parameters

for maximum growth and survival of micrografts. The standardized conditions for control shoots have been utilized for micrografting of nine putative transformants.

Successful graft unions established between two cultivars and between two species demonstrate a heterologous graft-union compatibility in cotton. The present micrografting method can be applied round the year and has several applications such as (1) obtainment of a high percentage of viable plants from *in vitro* regenerants generally difficult to root (2) recovery of whole plants from putative transformants which are usually extremely slow growing due to antibiotic stress or fail to root (3) acceleration of shoot growth (4) for understanding histochemical basis of successful graft-unions and graft rejection.

5.3.8. Southern analysis

Putative transformants analyzed with non-radioactive method of Southern to confirm the presence of NPTII gene were found to be negative. Southern analysis by radioactive method with only two surviving plants was carried out. Though, Southern analysis confirmed the integration of NPT II gene in cotton callus tissue (lane 5) and tobacco plant (used as a positive control) (lane 9), however, both the putative transformants of cotton cultivar NHH-44 (lane 7 & 8) did not show any bands. Absence of NPTII gene in putative transformants suggest that these are the escapes of the transformation event surviving on selection pressure for a period of 3 months. It is also evident from the figure (Fig. 5.14A) that transformed callus of cotton cultivar NHH-44 and transgenic tobacco treated with *Agrobacterium* strain pGV2260 harboring the p35SGUSINT received multiple copy of the NPT II gene.

Fig. 5.14

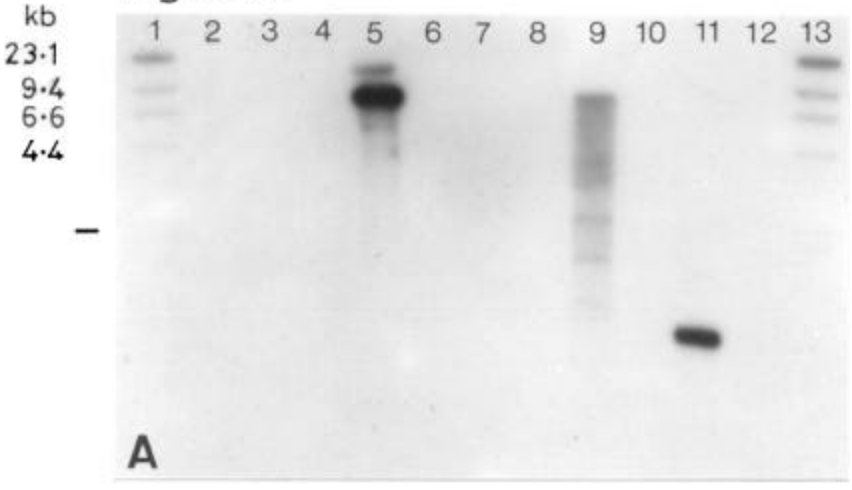


Fig. 5.14

Southern analysis: Agarose gel electrophoresis of *Hind*III digested DNA from - Control callus (lane 3), transformed callus (lane 5), putatively transformed plants of cultivar NHH-44 (lane 7 & 8) and transformed tobacco (lane 9). Lanes 1 & 13 are λ- DNA *Hind*III digest molecular weight maker. Lane 11 is the 0.85 kb fragment of NPTII from pHP23. Probe used $^{\alpha 32}$p labelled 0.85 kb NPTII from plasmid pHP23.

5.4. Conclusion

From the present studies, it can be concluded that frequency of transformation in cotton cultivar NHH-44 is largely influenced by the period and mode of treatment of *Agrobacterium tumefaciens* strain harboring plasmid p35SGUSINT. Of all the treatments tested, spotting of *Agrobactrium* suspension (S2) or dipping of explants in *Agrobactrium* suspension (S1) for a 3-4 seconds seems to be the ideal methods of treatment for affecting transformation in split embryo axes. The integration of GUS and NPTII genes in callus tissue has been confirmed both by histochemical and southern analysis. Intense GUS activity was observed in callus tissue and embryo axis. Putatively transformed shoots of cultivar NHH-44 though survived on selection medium for a period of 3 months, did no show any GUS activity and were Southern negative

The difficulty in growth and rooting of the putatively transformed cotton shoots was overcome by an *in vitro* micrografting method. *Ex vitro* micrografting though successful, had low survival of grafted shoots. Grafted plants on transfer to greenhouse and field conditions grew to maturity and set seeds. The micrografting protocol developed has potential use in recovery of transformed but slow growing and difficult to root microshoots and development of cotton transgenics.

The present study on transformation of cotton could be of immense importance as report on genetic transformation of Indian cultivars is presently not available.

Part of this work has been published as a paper entitled " Recovery of *in vitro* cotton shoots through micrografting" by Banerjee AK, Agrawal DC, Nalawade SM and KV Krishnamurthy. Current Science, Vol. 78 (5), 623 –626 (2000).

CHAPTER 6

TRANSIENT GENE EXPRESSION IN COTTON *VIA* PARTICLE BOMBARMENT METHOD

6.1. Introduction

After *Agrobacterium*-mediated transformation, particle bombardment (or Ballistics), wherein microscopic particles coated with genetically engineered DNA are explosively accelerated into plant cells (Klein *et al*. 1987; Sanford 1988) has become the second most widely used vehicle for development of transgenic plants (Rochange *et al.*1995). The technique has not only led to the efficient production of transgenic "model plants", but has also opened a route for genetic engineering of major crop plants (Potrykus 1995).

The technique involves accelerating microprojectiles (tungsten or gold) to velocities at which they can penetrate plant cell walls. This is a mechanical method of introducing DNA into almost any plant species and genotype (Hinchee *et al*. 1994). It may be highly advantageous when major biological barriers exist to either *Agrobacterium* or protoplast mediated transformation (Hinchee *et al.*1994).

6.1.1. History of Particle Bombardment

Mackenzie *et al*. (1966) developed the first generation of microprojectile technique for gene transfer into plant cells. They used high velocity microprojectiles to wound plant cells for facilitating the entry of viral particles or nucleic acids. Later, Sanford *et al* (1987) at Cornell University developed a range of devices to accelerate tungsten microprojectiles (1- 4 μm in diameter) to velocity at (250 m/s) sufficient to penetrate plant cell walls and membranes.

Since the high velocity microprojectiles carrying DNA or other substances is 'shot' into cells, it represent a biological ballistics and hence the term "biolistics" has been coined by its inventors (Sanford 1988). The technique has also been referred as particle bombardment, microprojectile bombardment, particle acceleration, the gene gun method or the particle gun method.

6.1.2. Types of Particle Guns

The potential of particle bombardment mediated transformation of plants and other organisms has stimulated commercialization of "Particle gun" and has inspired development of prototypes devices by several laboratories. Six main devices reported are as follows: 1. Biolistic PDS-1000/He 2. Electric discharge particle acceleration (ACCELL technology) 3. Particle Inflow Gun 4. Airgun device 5. Microtargeting device and 6. Helios gene gun system

Sanford *et al*. (1987) were the first to design a particle delivery system. The device employed a gunpowder charge to propel tungsten particles. Though the device was successful for genetic transformation of diverse plant species (Sanford 1990), lack of control over the power of the bombardment and substantial tissue damage limited the number of stable transformants. Significant technical improvement over the gunpowder device has been made into the recent model of PDS-1000/He marketed by Bio-Rad laboratories. The PDS-1000/He device is powered by a burst of helium gas that accelerates a macrocarrier, upon which millions of DNA coated microcarriers have been dried. Compared to the gun powder device, this system has several advantages such as: it is cleaner and safer, allows better control over bombardment power, distributes microcarriers more uniformly over target cells, is more gentle to target cells, is more consistent from bombardment to bombardment and increases the transformation frequency (Sanford *et al.* 1991).

The electric discharge particle acceleration (ACCELL technology) utilizes an instrument to accelerate DNA coated gold beads to a desired velocity by varying the input voltage. McCabe & Christou (1993) have described major advantages of this technique compared to other bombardment systems in terms of high transformation frequencies for major agronomic crops. This methodology has been used for genetic transformation of soybean (McCabe *et al*. 1988), cotton (McCabe & Martinell 1993), rice (Christou *et al.* 1991, 1992), corn, peanut and woody species. The technique allows control over particle penetration to specific cell layers and tissue damage through fine tuning.

The next device, Particle Inflow Gun (PIG) is based on acceleration of DNA- coated tungsten particles directly in a helium stream. Vain *et al.* (1993) have described this device in detail. Using this bombardment apparatus, high levels of transient expression of GUS gene in embryogenic suspension cultures of maize, soybean, leaf tissue of cowpea and stable transformation in soybean and maize have been obtained (Vain *et al.* 1993).

Air gun device for particle bombardment has been described by Oard (1993). In this device, compressed air in a cylinder of a air gun was used as source of propulsion for DNA coated tungsten or gold particles. The device has been used exclusively for transient gene expression experiments using the GUS marker.

Yet in an another approach, Sautter *et al*. (1991), Sautter (1993) has developed a microtargeting device for particle bombardment of meristems. In this system, instead of a

macroprojectile, microtargeing uses the law of Bernoulli for acceleration of highly uniform–size gold particle. It is ballistic approach which is particularly suitable for the controlled delivery of microprojectiles to meristem cells in situ. This way it delivers 80% of the particles to an area as small as 150 μm in diameter, which corresponds to the size of a meristem. Sautter *et al.* (1995) have summarized their experience with microtargeting of transgenes to wheat shoot apical meristems cells.

Recently, Bio-Rad laboratories has commercialized the "Helios gene gun". It is a hand-held biolistic device capable of delivering of DNA into any eukaryotic organisms. The unique device uses a helium pulse to accelerate gold microcarriers coated with nucleic acids into *in vivo* and *in vitro* targets. It provides rapid and direct gene transfer *in vivo*. The device is useful for gene therapy. In plants, meristematic tissues and leaves are obvious targets for *in vivo* transformation.

6.1.3. Applications of particle bombardment technology

Particle mediated gene transfer has been used in a variety of biological systems ranging from microbes to organelles, to agriculturally important plants and to animals. Several plant species belonging to both monocots and dicots such as maize (Fromm *et al*. 1990), soybean (McCabe *et al*. 1988) wheat (Vasil *et al*. 1992), cotton (Mc Cabe & Martinell 1993) and rice (Christou *et al.* 1991) have been successfully transformed.

Through this process, DNA has been reproducibly delivered into mitochondria in yeast (Johnston *et al.*1988) and chloroplasts in *Chlamydomonas* (Boynton *et al.* 1988).

Particle bombardment technology has been used in gene transfer to mammalian cell cultures and somatic tissues (Yang 1992). The advances made in the technology may benefit basic research and lead to possible industrial and chemical applications, notably human gene therapy. Particle bombardment provides a useful tool for *ex-vitro* gene transfer into a wide variety of surgically excised tissues and cells. It provides a new approach to evaluate somatic expression of therapeutic genes in various tissues *in vivo* (Yang & Swain 1994).

6.1.4. Advantages of Particle bombardment mediated transformation

Genetic transformation through particle bombardment offers several advantages (Gray & Finer 1993) over *Agrobacterium*-mediated transformation which are as follows:

1. The technique is capable of overcoming the host-range limitations of *Agrobacterium*
2. Non-hosts of *Agrobacterium*, such as monocots are most easily transformed

3. DNA can be delivered into any tissue
4. Transformation is genotype independent (Walden & Wingender 1995)
5. Plasmid construction is simplified, since DNA sequences essential for T-DNA replication and transfer in *Agrobacterium* are not required
6. The introduction of mulitple plasmids (cotransformaiton) is routinely accomplished with particle bombardment, such that a single large plasmid containing multiple transforming DNA sequences is not needed
7. False positives resulting from growth of *Agrobacterium* in host tissues are eliminated
8. Transformation protocols are simplified, since complex bacteria/plant inter relationships, that vary with each system are eliminated

6.1.5. Transient gene expression

In the process of transient gene expression, the microcarrier carrying DNA is delivered to the cytoplasm or to the nucleus. The genes adhering to microcarrier are then taken up by the nucleus and transcribed. This gene expression is termed as "transient gene expression". If the foreign gene is introduced into a sufficient number of cells, its expression can be quantitatively measured after 12-48 hrs of the bombardment. Several thousand copies of each plasmid (gene) can be resident of each microcarrier. Many of these copies contribute transient expression, however, only one to several copies are eventually integrated into the plant genome (Cooley *et al.* 1995). Blue spots obtained with GUS assay often are not confined to single cells. The size of the spots can vary considerably and the margin of the spots diffuse. Therefore such expression are referred as 'blue foci' (Vain *et al.*1993), 'GUS foci' (Bommineni *et al.* 1994), 'expression units' (Charest *et al.* 1993) or simply 'blue spots'.

In the present chapter, though we have used two plasmid constructs harboring reporter and selective markers, GUS (β-glucuronidase) expression in embryo axis after 72 hrs of bombardment has been termed as "transient gene expression".

6.1.6. Critical variables for particle bombardment

A number of factors are needed to be considered carefully for successful transformation through particle bombardment. The factors that interact to affect the frequency of transiently expressing cells in the bombardment tissues are : composition and size of the metal particles (microprojectiles), nature, form and concentration of DNA, DNA attachment to the microprojectiles prior to bombardment, impact velocity of the microprojectiles/DNA complex

and degree of tissue damage suffered on bombardment (Birch & Bower 1994). Apart fom these, environmental and biological factors also play an important role in transformation efficiency. Among the environmental factors, temperature, photoperiod and humidity of donor plants, explants and bombarded tissues may adversely affect the outcome of transformation process. Some explants require a healing period after bombardment under special regimens of light, temperature and humidity. Biological factors include choice and nature of explant, pre- and post bombardment culture conditions etc. (McCabe & Christou 1993).

6.1.7. Particle bombardment mediated transformation studies in cotton

The first report on particle bombardment mediated transformation in cotton was published by Finer & McMullen (1990). They bombarded embryogenic cell suspensions of Coker 310 with hygromycin genes using Dupont Biolistics™ Particle Delivery system (Model BPG). Hygromycin resistant transgenic plants were developed via somatic embryogenesis, five months after the bombardment.

In the next report, McCabe & Martinell (1993) have described the protocol for variety independent transformation in cotton. They bombarded meristems (embryo axes) using the electric discharge gun (ACCELL technology) for gene transfer. Integration of GUS gene was demonstrated in R0 and R1 transformants. Progeny analysis showed transmission of transgene in a Mendelian fashion. Chlan *et al.* (1995) introduced the (NPTII) gene into meristems of embryo axis explants of cotton using the Bio-Rad PDS/1000/He gene gun.

In an another report, Keller *et al*. (1997) developed transgenic plants resistant to bialaphos (a gene encoding phosphinothricin acetyltranferase). Meristems of the seed axes of elite varieties of cotton were bombarded using ACCELL technology. Integration of the bar gene in transgenic plants was confirmed by Southern's and gene expression was confirmed by northern blot analysis. The gene is inherited in a Mendelian fashion in the progenies of germline transformants.

More recently, Rajasekaran *et al*.(2000) have achieved high frequency stable transformation of cotton by particle bombardment of embryogenic cell suspension cultures of cultivars Acala B1654 and Coker 315. Transformation was carried out by using the PDS 1000/He biolistic device. They observed increased stable transformation frequency of 4% compared to 0.7% in an earlier report by Finer & McMullen (1990). The high efficiency of stable expression was due to the multiple bombardment of rapidly dividing cell suspension

cultures and the selection for transformation cells by gradually increasing the concentration of the antibiotic. Reports on particle bombardment mediated transformation in cotton have been given in Chapter 1 (Table 1.12).

From the review of literature, it is evident that transgenic cotton has been developed by particle bombardment method. The method has advantages compared to *Agrobacterium* in that it is genotype independent. Reports of transformation of Indian cultivars of cotton by both *Agrobacterium* and particle bombardment methods are not available so far. It could be due to recalcitrant nature of Indian cultivars under *in vitro* conditions. In the present chapter, we have made an attempt to optimize different parameters of particle bombardment method for transient gene expression in embryo axes of two important Indian cultivars of cotton.

6.2. Materials and Methods

6.2.1. Isolation of plasmid DNA from *Agrobacterium* strains pGV2260 (p35SGUSINT) and pBI 121 (pIBGUSINT).

Plasmid DNA from both the *Agrobacterium* strains was isolated by alkaline lysis method (Sambrook *et al*. 1989). Purification of DNA was carried out by phenol: chloroform method.

6.2.2. Transfer of plasmids (p35SGUSINT and pIBGUSINT) to *E.coli.*(HB101) through electroporation

This involves first the preparation of competent recipient cells (*E.coli*) and then transfer of plasmid DNA to *E.coli* by electroporation.

6.2.2.1. Competent cell preparation

E..coli (HB101) cells were grown in LB medium with vigorous shaking (200 rpm) at 37 °C to an A_{600} of 0.5 (Sambrook *et al*, 1989). The cells were harvested by chilling the flask briefly on ice and centrifuging at 5000 rpm for 10 min. at 4°C. Cells were washed with electroporation buffer (containing 272 mM sucrose, 7 mM Sodium phosphate buffer of pH 7.4 and 1 mM $MgCl_2$). Thereafter, washed cells were resuspended in an ice cold sucrose buffer in 1/20 of the original volume and kept in ice.

6.2.2.2. Electroporation

We used Bio-Rad Gene Pulser electroporation unit (Bio-Rad Laboratories, USA) (Fig. 6.1A). Electroporation was carried out according to the instructions given in the manual. Aliquots of 0.8 ml of competent cell suspension was pipetted out into 4 cuvettes. Plasmid DNA (2 μg) of p35SGUSINT was added to cell suspension in two cuvettes. DNA/cell suspension in cuvette were mixed by gentle pipetting. Other two samples without plasmid DNA served as controls. All four cuvettes were kept on ice for 30 min. Thereafter, electroporation was carried out at 2000, 2500 volts for varying periods of time. Duration of pulse was noted in each case. After application of the pulse, the sample in each case was diluted with 8 volumes of LB medium and incubated at 37 °C for 1 h. Aliquots of these samples were plated on LB medium containing Kanamycin 100 mg/l. The streaked plates were incubated at 37 °C for 72 hrs.

Similarly, electroporation of plasmid pIBGUSINT was carried out using the above mentioned procedure. However, in this case kanamycin (50 mg/l) was used as selective agent. Transformants (resistant colony) in both the case were selected after 72 hrs of incubation.

6.2.2.3. Analysis of plasmid DNA from transformed *E. coli*

To confirm the integration of the plasmid, the transformed cell lines of both the plasmids were grown in liquid LB medium containing antibiotic (kanamycin 100 mg/l for p35SGUSINT and 50 mg/l for pIBGUSINT) with vigorous shaking (200 rpm) at 37 °C. Plasmid DNA was isolated from both the *E.coli* strains and were digested with Eco R1 and Hind III. Thereafter, DNAs were electrophoresced and the band patterns were noted.

6.2.3. Isolation of plasmid DNA from *E.coli* (HB101) for particle bombardment

Plasmid DNA from both the *E. coli* cell lines harboring p35GUSINT and pIBGUSINT plasmids were isolated separately by using Sigma High pure TM plasmid preparation kit (Sigma, USA). DNA obtained was quantified.

6.2.4. Methodology of particle bombardment

PDS-1000/He (Bio Rad Laboratories) particle delivery system was used for the present study (Fig. 6.1B). Before carrying out particle bombardment, the following set up and stock solutions were prepared and kept ready**:** preparation of microcarrier, stock solutions of spermidine and Calcium chloride ($CaCl_2$), sterilization of accessories of PDS-1000/He, precipitation of DNA onto microcarrier, preparation of macrocarrier, preparation of explants. Details of these are given in the following sections:

6.2.4.1. Microcarrier preparation

1. Take 60 mg of gold particles (1.1 μm) in a microfuge tube.
2. Add 600 μl of 100 % ethanol to gold particles
3. Vortex on high for 2 min and centrifuge at 10,000 rpm for 1 min.
4. Remove the supernatant and repeat the step 2 & 3 thrice
5. Resuspend the particles in 1 ml of sterile distilled water and repeat the step 3
6. Remove the supernatant and repeat the step 5
7. Resuspend the microcarriers in 1 ml of sterile distilled water
8. Distribute aliquot (100 μl) of microcarrier suspension into 1.5 ml of microfuge tubes while vortexing.
8. Store aliquots at 4 °C.

Fig. 6.1

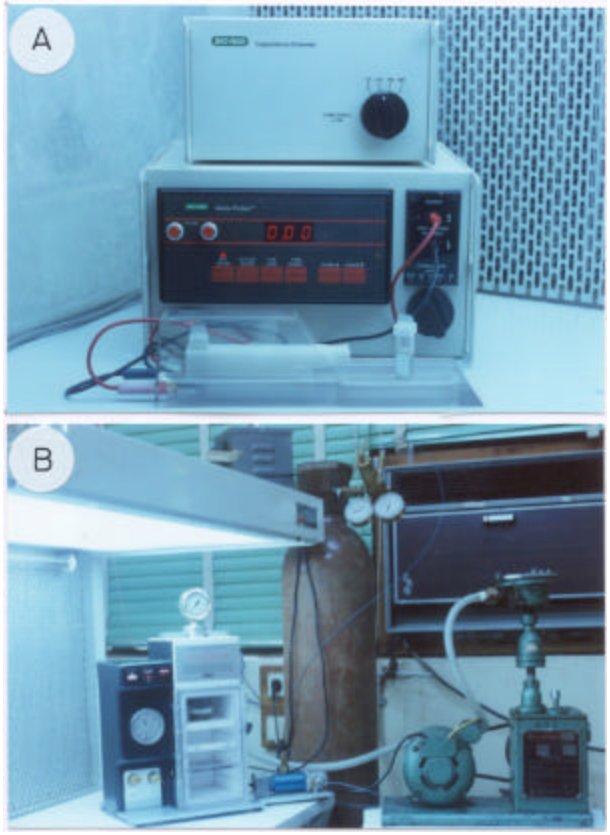


Fig. 6.1

A. Bio-Rad Gene Pulser™ unit used for transfer of *Agrobacterium* plasmids to *E.coli.*

B. Biolistic ® PDS – 1000/ He Particle Delivery System set up.

6.2.4.2. Preparation of 0.1 M spermidine stock

1. Dissolve 145 mg of spermidine (Sigma, USA) in 10 ml of sterile double distilled water
2. Aliquot 40 µl of spermidine solution into 1.5 ml of microfuge tubes.
3. Store at – 20 °C.

6.2.4.3. Preparation of 2.5 M $CaCl_2$ stock

1. Dissolve 1.837 g of Calcium chloride ($CaCl_2$, $2H_2O$) in 5 ml of double distilled water.
2. Filter sterilize and dispense 100 µl of aliquots in 1.5 ml of microfuge tubes.
3. Store at 4° C.

6.2.4.4. Sterilization of various accessories of PDS 1000/He gene gun

All the accessories of particle bombardment like macrocarriers, rupture disks, stopping screens, macrocarrier holders, macrocarrier cover lid were kept merged in 95 % ethanol for 2-3 hrs before the bombardment. After that these were spread on a sterile Whatman filter paper for drying in a laminar airflow cabinet. The bombardment chamber was sprayed with 95% ethanol. Rupture disk retaining cap, microcarrier launch assembly parts were swabbed with 95% ethanol and were allowed to dry in a laminar air flow cabinet.

6.2.4.5. Precipitation of DNA onto microcarriers

1. Add 10 µl of DNA (µg/µl) to 100 µl gold particles in 1.5 ml microfuge tube (as prepared in section 6.2.4.1).
2. Vortex for few seconds
3. Add 100 µl of $CaCl_2$, $2H_2O$ solution and mix by vortexing
4. Add 40 µl of spermidine solution and mix by vortexing for 3 min.
5. Allow microcarriers to settle to bottom of the tube for 10 min.
6. Carefully pipette out supernatant as much as possible and discard
7. Add 200 µl of absolute ethanol (HPLC grade) to the sedimented microcarriers.
8. Vortex briefly and centrifuge at 5000 rpm for few seconds.
9. Allow the particles to settle to bottom of the tube.
10. Pipette out supernatant carefully and discard.
11. Resuspend the particles in 120 µl of absolute ethanol.

6.2.4.6. Preparation of macrocarriers

1. Pipette 10 µl of plasmid DNA coated microcarriers (while continuous vortexing) and dispense in the center of each sterile macrocarrier (already placed in the holder).
2. Allow them to dry for about 5 min in a low relative humidity and vibration free environment (place holders with macrocarrier having DNA coated particles on a support, over $CaCl_2$ in a large closed petridish).

6.2.4.7. Explant preparation for the bombardment

Seeds of cotton cultivars NHH-44 and DCH-32 were germinated as described earlier (Chapter 3). Embryo axes were excised from germinated seeds and their radicles were discarded. Further, cotyledonary leaf bases were removed to expose the meristem. The explants were arranged (40 explants/dish) on a sterile filter paper moistened with 1 ml of MS1 medium (described in section 6.2.4.9) in a pre-sterilized plastic petridish (55 mm). The explants were arranged in a manner that their apical regions face the trajectory of DNA coated microcarriers. In a separate set, apical meristems of embryo axes were exposed by removing the cotyledonary leaf bases and leaf primordia under a dissection microscope. These were also used as explants and arranged as mentioned above.

6.2.4.8. Variables used for the bombardment

Two plasmids, p35SGUSINT (Chapter 5, Fig. 5.2B,) and pIBGUSINT (Fig.6.2A), gold microcarriers (1.1µm), rupture disks (1100,1300 psi), number of bombardments (once or twice) and target-cell distance (6, 8 cm) were used as variables in the study. For each bombardment, chamber vacuum was raised up to 28 Hg before firing.

6.2.4.9. Regeneration medium for embryo axes

Medium standardized earlier (Chapter 3) for induction of multiple shoots from embryo axes was used for regeneration of bombarded explants. Medium contained MS salts+B5 vitamin+BAP (0.1 mg/l)+NAA (0.02 mg/l)+sucrose (2%) and agar (0.65%). Hereinafter, this medium is referred as MS1 in this chapter.

6.2.4.10. Determination of lethal dose of kanamycin and phosphinothricin for embryo axes

The lethal dosage (LD_{50}) of kanamycin for embryo axes already determined earlier (chapter 5, section 5.2.5.1), has been used in the present study. The lethal dosage (LD_{50}) of phosphinothricin was determined by inoculating embryo axes on medium containing MS

satls+B5 vitamins+BAP (0.1 mg/l)+NAA (0.02 mg/l) +sucrose 2% and agar (0.65%) and supplemented with various concentrations of phosphinothricin (5, 10, 15, 20 mg/l). The cultures were incubated for four weeks at 30 °C under a 16 h photoperiod at a light intensity of 27 μE m^{-2} s^{-1} provided by cool white fluorescent tubes.

6.2.4.11.Culture of embryo axes after bombardment

After bombardment, the explants were kept in dark for 24 h. Thereafter, these were transferred to a semi-solid MS1 medium containing kanamycin (50 mg/l) or phosphinothricin (10 mg/l) and were incubated at 30 ^{0} C at a light intensity of 27 μE m^{-2} s^{-1}.

6.2.4.12. Transient expression in embryo axes (GUS assay)

For GUS assay, random samples of embryo axes were taken after 72 h of incubation. The procedure of GUS assay has been described in chapter 5. Blue loci, indicative of transient GUS expression, were counted 24 h after addition of the X-Gluc substrate solution under a sterioscopic microscope.

6.2.5. Histology of embryo axis

Embryo axes (with or without exposed apical meristem) were fixed in formalin : acetic acid : alcohol (5 : 5 : 90) and histology of these explant was carried out as described in chapter 2 (Materials and Methods).

Fig. 6.2

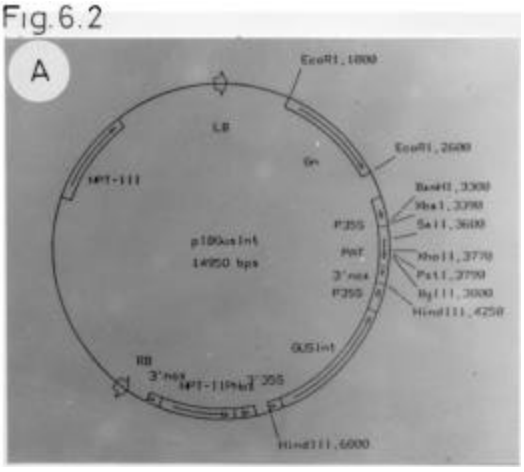


Fig. 6.2

A. Plasmid map of pIBGUSINT.

6.3. Results and Discussion

The present study was undertaken to ascertain the transfer and expression of reporter gene (GUS) in embryo axes of cotton cultivars NHH-44 and DCH-32 (*Gosypium hirsutum* L.) through biolistic method of plant transformation.

6.3.1. Isolation of plasmid DNA from *Agrobacterium* strains pGV2260 (p35SGUSINT) and pBI121(pIBGUSINT)

The yield of Plasmid DNA isolated from *Agrobacterium* strains was very low. Therefore, we decided to transfer these plasmids to *E.coli* strain (HB101) for ease of isolation and high yield of plasmid DNA.

6.3.2. Transfer of plasmids (p35SGUSINT and pIBGUSINT) to *E.coli.*(HB101) through electroporation

The use of high voltage electroporation is an effective method for introducing genetic material into eukaryotic (Potter *et al.* 1984) as well as procaryotic (Dower *et al.* 1988) cells. Electroporation, in which a brief high voltage electric discharge is used to render cells permeable to DNA, has revolutionized the transformation of bacteria. The technique is fast, simple, reproducible, frequently gives very high transformation frequency and is applicable to a wide

range of bacterial types including *E. coli* (Chassy 1988). Direct transfer of bacterial plasmid DNA by electroporation and transformation of E.coli have been reported (Dower *et al.* 1988 ; Summers & Withers 1990).

We noted survival of a few kanamycin resistant individual colonies of both the *E.coli* strains (harboring plasmids p35SGUSINT and pIBGUSINT) on selection medium having kanamycin 100 and 50 mg/l respectively. This was achieved when both the plasmid DNA were subjected to electroporation for 3.8 msec pulse at 2000 volts. Cells did not survive in control sets and also when electroporation was carried out at 2500 volts. Resistant colonies grew vigorously in liquid LB medium containing kanamycin (100 mg/l for p35SGUSINT and 50 mg/l for pIBGUSINT). In this experiment, we did not record the frequency of transformation in both the *E.coli* strains.

In the present experiment, our aim was limited to the transfer of *Agrobacterium* plasmids to *E.coli* strains for higher yields of DNA needed for bombardments. Therefore, a critical study on parameters of electroporation was not carried out.

6.3.3. Analysis of plasmid DNA from transformed E.coli cells

Gel electrophoresis of plasmid DNA (p35SGUSINT) isolated from the transformed *E.coli* strain exhibited the expected bands (Fig. 6.3A) after digestion with HindIII or Eco R1 (Also refer plasmid map, Fig.5.2B, chapter 5). Similarly, the integration of other plasmid DNA (pIBGUSINT) to *E.coli* was also confirmed.

6.3.4. Isolation of plasmid DNA (p35SGUSINT & pIBGUSINT) from *E.coli* (HB101) for particle bombardment.

The yield of plasmid DNA (p35SGUSINT and pIBGUSINT) from *E.coli* isolated by Sigma High pure TM plasmid preparation kit (Sigma, USA) was very high (around 3 µg /ml of cells). DNA obtained was pure in quality. This highly pure DNA was used for particle bombardments.

6.3.5. Determination of lethal dose of kanamycin and phosphinothricin for embryo axes

Explants on basal medium without phosphinothricin (controls) developed normal shoots after 3 weeks of incubation. Embryo axes cultured on medium with phosphinothricin 5 mg/l showed initial shoot development. However, concentrations 10 mg/l and higher were lethal and

inhibited the shoot development completely (Fig. 6.4A). Hence, phosphinothricin 10 mg/l was taken as the selection pressure for the explants bombarded with plasmid pIBGUSINT.

6.3.6. Transient gene expression in embryo axes (Gus assay)

In the present study, efforts have been made to optimize some of the parameters used in particle bombardment method for efficient transient gene expression in embryo axes of two important Indian cultivars of cotton. Though we have used plasmid constructs harboring selective markers for stable transformation, expression of GUS in embryo axes after 72 hrs of bombardment has been termed as "transient gene expression" in this chapter.

For any plant tissue that is used for the first time for particle bombardment, optimal parameters for transient or stable gene expression are necessary to be established. It is because transient expression of introduced gene can be studied within the first few days after bombardment. Also it is very useful for optimizing the variables affecting the efficiency of DNA transfer through bombardment of microprojectiles (Schopke *et al.* 1997).

Fig. 6.3

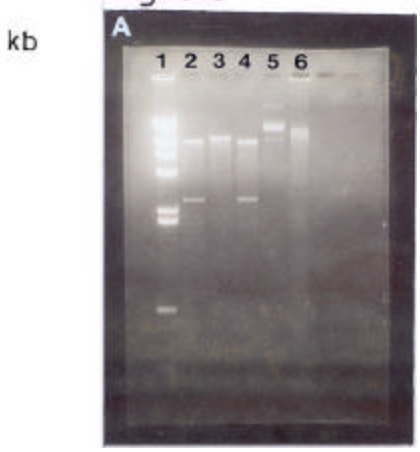


Fig. 6.3

A. Agarose gel electrophoresis of plasmid DNA isolated from *E. coli* and *Agrobacterium* strains harboring plasmid p35GUSINT. Lane 1- λ- DNA *Hind*III digest molecular weight marker; lane 2 & 4- *Hind*III digested plasmid DNA (*E .coli*); Lane 3- *EcoR*1 digested plasmid DNA (*E.coli*); Lane 5- Undigested plasmid DNA (*E.coli).* Lane 6- undigested plasmid DNA (*Agrobacterium).*

Fig.6.4

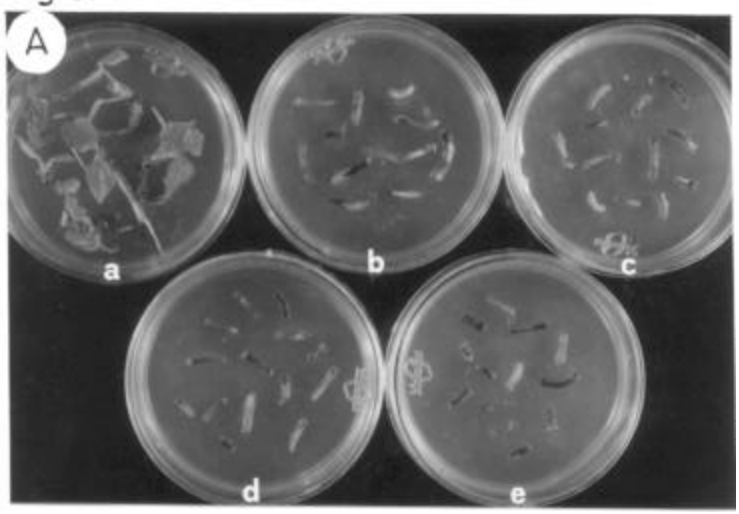


Fig. 6.4

A. Determination of lethal dosages of phoshphinthricin for embryo axes: a=control, b=5 mg/l, c=10 mg/l, d=15 mg/l, e=20 mg/l.

6.3.6.1. GUS Expression after single bombardments

Between the two constructs tested with cultivar NHH-44, p35SGUSINT yielded the maximum percentage of explants showing GUS expression (29.16%) irrespective of target distance and rupture disks used in the present experiment (Table 6.1). The plasmid pIBGUSINT on the other hand resulted in the lowest percentage (12%) of explants having blue loci when bombarded at a target distance of 6 cm with 1100 psi rupture disks. Blue loci were observed in different regions of the embryo axes (Fig. 6.5 B-E). Embryo axes of cultivar DCH-32 when bombarded with gold microcarriers coated with plasmid p35SGUSINT using 1100 psi and 6 cm target distance resulted in 23.68% of GUS expression, while 25.80% of explants showed GUS expression when bombarded with 1300 psi rupture disk and at 8 cm target distance. Since pIBGUSINT with cultivar NHH-44 resulted in the minimum percentage of

explants (only 12%) showing GUS expression, the construct was not used with cultivar DCH-32.

Gold microcarriers were used in the present study since these have been used in earlier reports on development of cotton transgenics through particle bombardment (McCabe & Martinell 1993; Chaln *et al.* 1995; Keller *et al.* 1997).

6.3.6.2. GUS expression after double bombardments

In the previous experiment, out of two cultivars tested, cultivar NHH-44 resulted in the maximum explants showing GUS after single bombardment. Hence, further experiments were carried out with NHH-44 alone to determine the effect of double bombardment keeping all other variables (target distance 6 cm, rupture disk 1100 psi) as constant.

Double bombardment of gold microcarriers coated with plasmid p35SGUSINT mariginally decreased the percentage of explants showing GUS expression (26.82%) compared to single bombardment (29.16%) (Table 6.1, 6.2). However, with plasmid pIBGUSINT an increase in the percentage of explants (from 12% to 19.04%) having blue loci was observed (Table 6.1, 6.2). In a few explants, GUS expression was observed in the apical meristem (Fig. 6.5 A).

In an earlier study with rice, Jain *et al*. (1996) obtained higher frequency of GUS expression by using gold microcarriers compared to tungsten. Gold microcarriers are generally preferred for biolistic bombardment due to their size uniformity, spherical shape, inert nature and non-toxicity to plant tissues.

Fig. 6.5

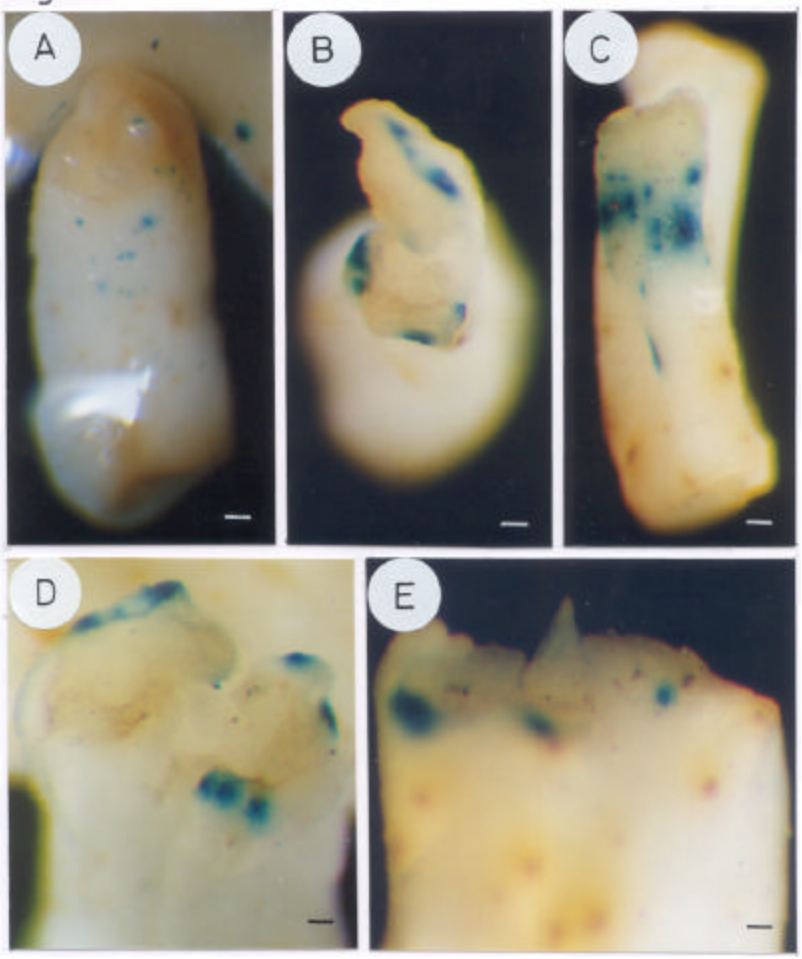


Fig. 6.5

A. GUS expression in embryo axes bombarded twice with gold particles coated with plasmid p35SGUSINT (bar=286 μm).

B– E. GUS expression in different regions of embryo axes bombarded with gold particles coated with plasmid p35SGUSINT (B=370 μm; C=400 μm; D=198 μm; E=162 μm).

There are several reports on integration of chimeric genes in plants via particle bombardment mediated transformation. Due to high regeneration potential and ease in tissue culture, embryo axis has earlier been used as an explant for gene transfer by particle bombardment method in soybean (McCabe *et al*. 1988), peanut (Schnall & Weissinger 1993), cotton (McCabe & Martinell 1993; Chlan *et al.* 1995) and sunflower (Hunold *et al*. 1995). Transient gene expression and optimization of different parameters of particle bombardment has also been reported in pearl millet (Taylor & Vasil 1991), Cassava (Schopke *et al*. 1997), sunflower (Hunold *et al*. 1995), maize (Vain *et al*. 1993), rice (Jain *et al*. 1996) and peanut (Lacorte *et al.* 1997).

Table 6.1: Transient GUS expression in embryo axes after single bombardments.

Cotton Cultivar	No. of E.axes bombarded	MC	Plasmid DNA	RD (psi)	TCD (cm)	No. of axes tested for GUS assay	No. of axes showing GUS expression	% of axes showing GUS expression
NHH-44	960	G	p35S GUSINT	1300	8	29	6	20.68
NHH-44	1275	G	p35S GUSINT	1100	6	48	14	29.16
NHH-44	1450	G	pIB GUSINT	1100	6	25	3	12.00
DCH-32	1060	G	p35S GUSINT	1100	6	38	9	23.68
DCH-32	840	G	p35S GUSINT	1300	8	31	8	25.80

MC- Microcarrier, G - Gold, TD- Target distance, RD- Rupture disk

Table 6.2: Transient GUS expression in embryo axes of cultivar NHH-44 after double bombardments.

No. of E.axes bombarded	MC	Plasmid DNA	RD	TD	No. of axes tested for GUS assay	No. of axes Showing GUS expression	% of axes showing GUS expression
594	Gold	p35S GUSINT	1100	6	41	11	26.82
300	Gold	pIB GUSINT	1100	6	21	4	19.04

MC- Microcarrier, RD- Rupture disk, TD- Target distance.

6.3.7. Culture of embryo axes after single bombardments

As in the present study, incubation of bombarded explants in dark for 24 hrs before they are shifted to light is considered necessary for the healing of tissues (McCabe & Martinell 1993; Chlan *et al.* 1995).

Embryo axes from cultivars NHH-44 and DCH-32 developed single shoots 80% and 75 % respectively when bombarded with gold microcarriers coated with plasmid p35GUSINT, 1100 psi rupture disks, 6 cm target distance and after two weeks of incubation. Out of 1275 embryo axes of NHH-44 bombarded, only 7 survived on kanamycin selection medium and induced shoots (Fig. 6.6A). In case of cultivar DCH-32, out of 1060 explants only 5 survived and induced shoots after three months. Leaf samples from these shoots were found to be GUS negative. Embryo axes when bombarded with plasmid pIBGUSINT under identical conditions though developed single shoots on basal medium supplemented with phosphinothricin (10 mg/l), turned brown after one month of culture (Table 6.3). Explants from cultivars NHH-44 and DCH-32 when bombarded with gold microcarriers coated with plasmid p35GUSINT, 1300 psi rupture disks, 8 cm target distance did not survive after three transfers to medium containing kanamycin selection pressure (50 mg/l).

6.3.7.1. Culture of embryo axes after double bombardments

In a separate experiment, bombardment of explants with gold particles twice decreased the shoot formation from embryo axes. Explants from cultivar NHH-44 developed single shoots (44.94%) when bombarded twice with gold microcarriers coated with plasmid p35GUSINT, 1100 psi rupture disks, 6 cm target distance and after two weeks of incubation. Out of 594 explants bombarded, only 2 survived and developed single shoots. The leaf samples of these shoots were found to be GUS negative. Embryo axis explants when bombarded with plasmid pIBGUSINT under identical conditions but cultured on basal medium containing phosphinothricin (10 mg/l) developed single shoots (46.33%) after 2 weeks of incubation. On further transfer of these shoots to fresh selection medium, shoots turned brown and died (Table 6.4).

Fig. 6.6

A. Shoots surviving on kanamycin (50mg/l).

Fig 6.6

Fig. 6.6.

A. Shoots surviving on kanamycin (50 mg/l)

Shoots which survived on selection pressure both from single or double bombardments could be escapes of transformation event since none of them showed GUS expression. In an earlier study on cotton transformation, McCabe & Martinell (1993) have reported that frequency of transformation differs among the cultivars and the number of germline plants produced was one per one thousand bombarded explants. In this report, plants were screened for GUS gene activity and GUS positive buds in the axils of transformed leaves were forced to develop into plants by pruning away non-transformed primary shoot tips. A similar approach was used by Keller *et al*. (1997) to develop cotton transgenics. This process resulted in two types of transformants: epidermal and germline. Even though stable integration and expression of the transgene occurred in both types of transformants, however, the progeny has not inherited the transgene if the epidermal cortex cell layer of a plant is only transformed. In our study, though we could achieve GUS expression in embryo axis explants, stable transformation in plants has not been achieved.

Table 6.3: Development of shoots from embryo axes after single bombardment.

Cotton Cultivar	No. of E. axes bombarded	MC	Plasmid DNA	RD (psi)	TD (cm)	No. of axes developed single shoots after 2 wks	% of axes developed single shoots	No. of shoots survived on kanamycin up to 12 wks
NHH-44	960	G	p35S GUSINT	1300	8	711	74.06	-
NHH-44	1275	G	p35S GUSINT	1100	6	1020	80.00	7
NHH-44	1450	G	PIB GUSINT	1100	6	1015	70.00	-
DCH-32	1060	G	p35S GUSINT	1100	6	795	75.00	5
DCH-32	840	G	p35S GUSINT	1300	8	590	70.23	-

MC- Microcarrier , G- Gold, RD- Rupture disk; TD- Target distance

Table 6.4: Development of shoots from embryo axes after double bombardment.

Cotton Cultivar	No. of E.axes bombarded	MC	Plasmid DNA	RD (psi)	TD (cm)	No. of axes developed single shoots after 2 wks	% of axes developed single shoots	No. of shoots survived on kanamycin Up to 12 wks
NHH-44	594	G	p35S GUSINT	1100	6	267	44.94	2
NHH-44	300	G	pIB GUSINT	1100	6	139	46.33	-

MC- Microcarrier, G- Gold, RD- Rupture disk; TD- Target distance

6.3.8. Histology of the embryo axis

Histology of the cotton embryo axis revealed that apical meristem covered with two primordial leaves is overgrown by cotyledonary leaf bases (Fig. 6.7 A-C). Therefore for particle bombardment mediated transformation of cotton embryo axis, it is desirable that tissues surrounding the apical meristem be removed so that the dome is directly exposed to the shower of DNA coated particles.

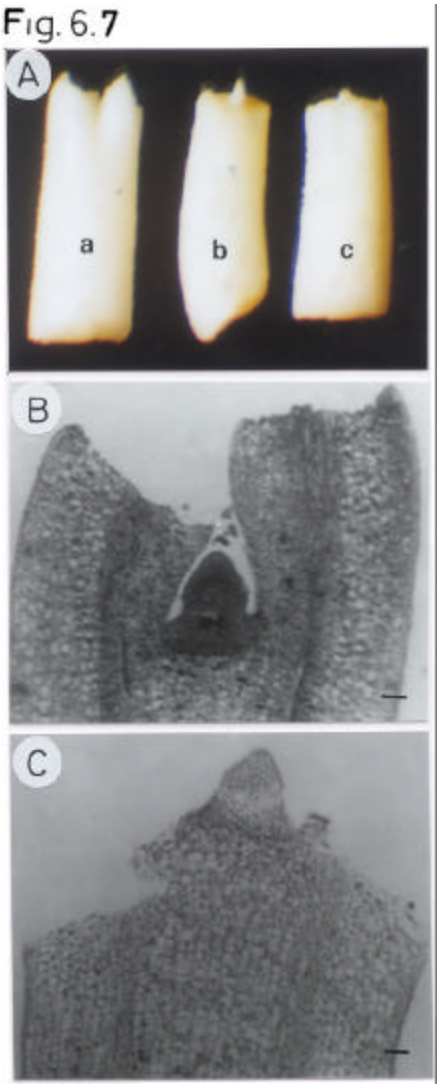


Fig. 6.7

A. Embryo axes devoid of cotyledons and radicles; a=unexposed apical meristem, b= apical meristem with leaf primordia attached; c=exposed apical meristem without leaf primordia).

B. Longitudinal section (l.s) of embryo axis with unexposed apical meristem (bar=65 μm).

C. Longitudinal section (l.s) of embryo axis with exposed apical meristem (bar=63 μm).

6.4. Conclusion

"Transient gene (β-glucoronidase) expression" by particle bombardment in embryo axes of cotton cultivar NHH-44 and DCH-32 has been achieved with two plasmids (p35SGUSINT and pIBGUSINT) independently. The maximum number of explants (29.16%) showing GUS expression was observed in cultivar NHH-44 with 1100 psi rupture disk at 6 cm target distance followed by DCH-32 (25.80%) with 1300 psi rupture disk at 8 cm target distance

Shoots survived on selection pressure both from single or double bombardments could be escapes of transformation event as none of the leaves of plants showed GUS expression. Though we could achieve "transient GUS expression", stable transformation in plants has not been achieved.

The present study demonstrates the usefulness of plasmids (p35GUSINT and pIBGUSINT) in the transfer of β-glucuronidase gene into cotton embryo axes through biolistic method. The results should facilitate the use of particle bombardment study of gene regulation in cotton by identifying factors that influence levels of transgene expression.

SUMMARY

Genetic engineering of plants, an integral component of plant biotechnology is one of the dynamic frontier of life sciences with rapidly emerging practical applications. It has the potential to provide solutions to a range of problems like pest control, biotic and abiotic stresses and increase in productivity.

Cotton is one of the most important commercial crops of the world valued for its fiber, oil and other by-products. It belongs to the genus *Gossypium* under the family Malvaceae. The crop is grown in 70 countries and about 180 million people around the globe are involved with the fiber industry which produces raw cotton worth 20–30 billion US dollars.

In India cotton is cultivated in an around 9.1 million hectares in three agroclimatic zones (Northern, central and southern zones). The crop provides the means of livelihood to an estimated 60 million people in the country. Although India is one of the major cotton producing countries in the world, insect menace still remains a serious concern. Out of total chemical pesticides used in crop protection in the country, almost 50% of them are required for cotton crop alone. Recently, cotton transgenics containing insecticidal protein genes of *Bacillus thuringiensis* var. *kurstaki* Cry 1A(b) and Cry 1A(c) have been developed and commercialized in the USA and a few other countries. Since India occupies a position among the top five cotton producing countries of the world, concerted efforts are being made to develop transgenic cotton of Indian cultivars.

An efficient plant regeneration protocol is a major pre-requisite for development of transgenic plants. At the time of initiation of this study, there was no report on *in vitro* plant regeneration from juvenile explants of Indian cutlivars of cotton. Therefore, this study was initiated with an objective of developing a highly reproducible and efficient *in vitro* plant regeneration method from explants with pre-existing meristems. The study also aimed to standardize *Agrobacterium* mediated transformation of cotton explants and their molecular characterization. Yet another objective of the study was to optimize different parameters of particle bombardment method for transient gene expression in embryo axis explants as an alternate approach of plant transformation so that Indian cultivars of cotton with agronomically desirable traits could be evolved through biotechnological methods.

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

A: *In vitro* induction of multiple shoots and plant regeneration from cotyledonary node and zygotic embryo axis explants of cotton

1. A protocol of plant regeneration from cotyledonary nodes of cultivar LRK-516 has been developed.
2. Induction of multiple shoots was achieved from cotyledonary nodes devoid of cotyledons and apical meristems. Explants from 35-day-old seedlings yielded the maximum number of shoots (4.7 shoots/explant) on Murashige and Skoog's (MS) basal medium supplemented with BAP and kinetin (2.5 mg/l each).
3. Explants from 35-day-old seedlings raised in glass bottles produced a higher number of multiple shoots (8.3 shoots/explant) than those grown in glass tubes and cultured on the same shoot induction medium**.**
4. Elongation of multiple shoots was obtained on liquid or agar MS basal medium without phytohormones.
5. Rooting of i*n vitro* shoots was achieved on half-strength semisolid MS basal medium supplemented with NAA (0.1 mg/l). Tissue culture plantlets survived (95%) after hardening under greenhouse conditions.
6. Induction of multiple shoots from embryo axes of three cultivars viz. NHH-44, LRK-516 and H-8 was achieved on a medium containing MS salts+ B5 vitamins, BAP (0.1) and NAA (0.02 mg/l). Further shoot proliferation was achieved on transfer of multiple shoot bunches to the fresh medium and on incubation for 10 weeks.
7. Rooted shoots survived (92%) after hardening in greenhouse and grew to maturity (100%) after transfer to field.

[*Part of this work has been published and also has been filed as an Indian patent - See author's publication section*].

B. Effect of antibiotics on shoot growth of zygotic embryo axis explants of cotton

In our transformation experiments with cotton embryo-axes, cefotaxime was incorporated in the medium to eliminate *Agrobacterium*. However, it was observed that when embryo-axes (which were not co-cultivated with *Agrobacterium*) were inoculated on the medium with cefotaxime as controls, it resulted in higher frequency of shoot

development. Hence this study was carried out to examine the effects of cefotaxime and three other antibiotics on embryo axes of cotton cultivars and their possible role in high frequency development of shoots.

1. Of the four antibiotics tested, cefotaxime, carbenicillin and chlroramphenicol at certain concentrations had a positive influence on the frequency of shoot development from embryo axes.
2. Streptomycin at all concentrations tested for cultivar NHH-44 had a negative effect on the frequency of shoot development.
3. The results obtained in the present study demonstrate that out of four antibiotics tested, cefotaxime is the best antibiotic for high frequency shoot development from embryo axis of cotton.

[*Part of this work has been published and also has been filed as an Indian patent - See author's publication section*].

C. *Agrobacterium tumefaciens* mediated transformation studies in cotton

The present study was carried out with the objectives of optimization of cocultivation with *Agrobacterium* to achieve transformation in cotton cultivars through embryo axes and standardization of an *in vitro* micrografting procedure for recovery of putative transformants.

1. Transformation frequency of cotton cultivars tested is largely influenced by the period and mode of treatment of *Agrobacterium tumefaciens* strain harboring plasmid p35SGUSINT.
2. Of all the treatments tested, spotting of *Agrobacterium* suspension or dipping of explants in *Agrobacterium* suspension for 3-4 seconds are found to be optimum.
3. Intense GUS activity was observed in callus tissue and embryo axis. The integration of GUS and NPTII genes in callus tissue has been confirmed both by histochemical and Southern analysis.
4. Putatively transformed shoots of cultivar NHH-44 though survived on selection medium for a period of 3 months did not show any GUS activity and were Southern negative
5. The difficulties encountered in rooting of the putatively transformed cotton shoots was overcome by an *in vitro* micrografting method.

6. *Ex vitro* micrografting though successful, had low survival of grafted shoots.
7. Grafted plants on transfer to greenhouse and field conditions grew to maturity and set seeds.

[*Part of this work has been published - See author's publication section*].

D. Transient gene expression in cotton via particle bombardment method

This study was carried out to optimize different parameters of particle bombardment method for transient gene expression in embryo axis of two cotton cultivars.

1. Transient gene (β-glucoronidase) expression in embryo axes of cotton cultivar NHH-44 and DCH-32 was achieved with two plasmids (p35SGUSINT and pIBGUSINT) independently.
2. The maximum number of explants (29.16%) with GUS expression was in cultivar NHH-44 followed by DCH-32 (25.80%) with gold microcarriers.
3. Shoots survived on selection pressure from single or double bombardments. These could be escapes of transformation event as none showed GUS expression.

The work presented in this thesis forms the basic frame work for biotechnology of cotton - the standardized regeneration protocol from cotyledonary node and embryo axis has application in genetic transformation of Indian cultivars of cotton. The positive role of cefotaxime in high frequency shoot development from embryo axes could be utilized gainfully in *Agrobacterium* mediated transformation to recover a large number of transformants during post co-cultivation studies. The results obtained from *Agrobacterium*-mediated transformation study could be utilized in ongoing efforts in development of transgenic cotton. *In vitro* micrografting protocol will be of immense use in recovery of slow growing and difficult to root transformants. Our initial results with particle bombardment on transient gene expression should facilitate the study of gene regulation in cotton by identifying factors that influence levels of transgene expression.

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