Plant regeneration from pre-existing meristems, embryo axis and mature explants of Indian cotton cultivars (*Gossypium hirsutum* L.)

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Plant regeneration from pre-existing meristems, embryo axis and mature explants of Indian cotton cultivars (*Gossypium hirsutum* L.)

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Dedicated

to my Father

Late. Shri Manohar D. Nalawade

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Date:
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CERTIFICATE

This is to certify that the work incorporated in the thesis entitle "**Plant regeneration from pre-existing meristems, embryo axis and mature explants of Indian cotton cultivars (*Gossypium hirsutum* L.)"** submitted by Mr. Satish Manohar Nalawade was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. K. V. Krishnamurthy)

Research guide

Pune

Date:

Key to abbreviations

ABA	Abscisic acid
$AgNO_3$	Silver Nitrate
B_5	Gamborg's medium (1968)
BA	6-Benzyl amino purine
cv.	Cultivars
^0C	Degree Celsius
2,4 - D	2, 4-Dichlorophenoxyacetic acid
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
GA_3	Gibberellic acid
Glu	L – Glutamine
Kin	Kinetin (6-furfuryl amino purine)
MS	Murashige and Skoog medium (1962)
NAA	α-Naphthaleneacetic acid
PDS	Particle Delivery System
Pic	Picloram (4Amino-3,4,6-trichloro picolinic acid)
2,4,5 – T	2,4,5-Trichlorophenoxyacetic acid
TDZ	Thidiazuron (1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea
Vol/Vol	Volume/volume (concentration)
Wt/Vol	Weight/ volume (concentration)

Synopsis

Synopsis

Cotton is one of the major economic crops of India and country is one among the top five cotton producers in the world. Cotton is an important natural source of fiber for textiles. Cotton has multifarious uses. Besides lint (fiber), cottonseed is a valuable source of edible oil, cake and meal for livestock feeds. Cotton belongs to genus *Gossypium* of the family Malvaceae. Of the 50 species of *Gossypium*, only four are domesticated. Among the four cultivated species, *G. aroboreum* and *G. herbaceum* are diploids and are known as 'Old World' cottons. The other two species, *G. hirsutum* and *G. barbadense* are allotetraploids and are known as 'New World' cottons. Cotton is grown in 90 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).

To meet the increasing demands of superior quality cotton by textile industry and also to restrict the damage caused to environment by excessive use of pesticides, there has been an increased interest in quantitative and qualitative improvement of cotton varieties. Conventional breeding techniques have limitations to meet these objectives, often due to incompatibility barriers between cotton species. Recently, biotechnological methods have been used to overcome this limitation and to introduce foreign genes in cotton (Chlan et al., 1995; Mc Cabe nad Martinell 1993). However, an efficient plant regeneration protocol is an essential pre--requisite for the development of transgenic plants. The majority of the reports published mainly from the USA are on Coker varieties that are not cultivated in India.

The present work entitled "**Plant regeneration from pre-existing meristems, embryo axis and mature explants of Indian cotton cultivars (*Gossypium hirsutum* L.)**" was undertaken to develop an efficient plant regeneration protocol using various explants derived from seeds, *in vitro* raised seedlings and field grown mature plants. Yet another objective of the work was to study application of explants with pre-existing meristems for genetic transformation in cotton by particle bombardment method.

CHAPTER 1. GENERAL INTRODUCTION

This chapter includes the introduction of the genus cotton (*Gossypium hirsutum L.*) and a through literature survey on *in vitro* regeneration and genetic

transformation studies in cotton. The present status of the cotton crop in terms of its production, area under cultivation has been described in the chapter. The aims and objectives of the present work are also envisaged in this chapter.

CHAPTER 2. MATERIALS AND METHODS

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

CHAPTER 3. *IN VITRO* PROPAGATION AND PLANT REGENERATION FROM EMBRYO AXIS EXPLANTS

A simple and rapid method of obtaining plants from explants derived from decotyledonated embryo axis has been described in this chapter. The chapter describes the effect of activated charcoal and incubation temperatures on shoot formation from embryo axis explants. The effect of phytohormones on induction and proliferation of multiple shoots in these explants has been described. *In vitro* rooting of shoots and hardening of plants has also been described in this chapter.

CHAPTER 4. PLANT REGENERATION FROM EXPLANTS CONSISTING OF PRE-EXISTING MERISTEMS FROM *IN VITRO* GROWN SEEDLINGS

The regeneration potential of various explants from in vitro grown seedling has been evaluated. This chapter describes the influence of the phyo-hormones, explant type, age of the explants, carbohydrate sources and the culture vessels in the induction and proliferation of multiple shoots. In vitro rooting of shoots obtained and hardening of plantlets have also been described in this chapter.

CHAPTER 5. CLONAL PROPAGATION USING NODAL SEGMENTS FROM FIELD GROWN MATURE PLANTS

In this chapter, plant regeneration protocol using nodal segments of field grown plants has been described. The protocol for induction, proliferation and maintenance of the shoot cultures from the accessory buds present in the axils of nodal segments of field grown mature plants has been described. The influence of type of culture vessel (test tube and conical flask) in proliferation of the multiple

shoots has been studied. *In vitro* rooting of shoots and acclimatization of the plants in soil have been described.

CHAPTER 6. APPLICATION OF EMBRYO AXIS EXPLANTS IN TRANSFORMATION OF COTTON *VIA* PARTICLE BOMBARDMENT APPROACH

This chapter describes the effect of different parameters of particle bombardment like rupture discs, type of microcarrier, target cell distance etc. on expression of β-glucuronidase (GUS) gene in embryonic axis explants. Attempts on the effect of phyto-hormones on morphogenesis in the transformed callus have been described.

Chapter 1

General Introduction

Introduction

1. The crop

Cotton is one of the most important multipurpose crops valued for its lint (fiber), oil, seed meal, hulls and several other by-products. The cultivated and wild varieties of cotton belong to genus *Gossypium* under the family Malvaceae. There are 50 species of *Gossypium* of which only four are cultivated. The two cultivated species *G. aroboreum* L. and *G. herbaceum* L. are diploid (2n=26) and are referred as "Old world" cottons. These account for less than 2 % of world cotton production due to varying fiber quality and low yield. *G. arboreum* L. is grown in India and *G. herbaceum* L. is cultivated in drier areas of Africa and Asia. The other two species *G. hirsutum* L. (upland cotton) and *G. barbadense* L. (extra long staple Egyptian or pima cotton) are allotetraploids (2n=52) and are referred as "New world" cottons (Lee 1984). These species evolved adventitiously by chromosome doubling of African and South American diploid species (Cherry *et al.* 1970). About 90 % cotton grown worldwide is *G. hirsutum* L. and the remaining 8 % accounts for *G. barbadense* L (Lee 1984). Cotton is grown in 90 countries on 32.6 million hectares 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 is indigenous to south and southeast Asia and has been cultivated in the Indus valley for more than 5000 years. Relics of Mohen-jo-daro period indicate a high degree of art in spinning and weaving with cotton at that time. India possessed a flourishing export trade in cotton and cotton fabrics in early historic times. Also cotton was indigenous to the America and was grown for clothing in Brazil, Peru and Mexico long before discovery of the America (Poehlman & Borthakur 1969). 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).

1.2. Distribution

The crop has a worldwide distribution covering all ranges of temperate, warm and tropical regions. It is annual, biennial or perennial in temperate zones, while its habit is one of tall shrubs or trees in tropics. Under cultivation, it is generally annual, except in some american

countries where it is still treated as perennial and grown in the field for about seven years (Sethi *et al.* 1960).

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 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). In India the cotton growing areas fall within 8° to 32° N latitude and 70° to 80° E longitude (Basu 1990). The top ten cotton growing countries, their area harvested, production and yield per hectare have been given in Table 1.1.

Table 1.1: Important cotton growing countries in the world.

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

(Source: F.A.O. Production Year Book 1997.)

1.3. Ecology

Cotton, a woody perennial shrub is a sun-loving plant and cannot tolerate shade especially in the seedling stage. Reduced light intensity due to prolonged overcast weather, shading from interplanted crops or too dense a stand retard flowering and fruiting and increases

boll shedding. The optimum temperatures for the germination of seed, seedling growth and further vegetative growth are 90°F, 75-85°F and 90°F respectively. Soil aeration, moisture and temperature are important factors in germination and early plant growth (Purseglove 1988). Lower temperature favors the production of vegetative branches and extends the cropping period where as high temperature increases the number of fruiting branches and reduces the cropping period. In areas where it is grown as rain-fed crop, the average rainfall usually is about 40-60 inches. Adequate moisture is required for early vegetative growth. First flowering period requires relative dryness, otherwise excessive boll shedding ensues. High moisture is required for boll swelling and renewed growth, followed by dry weather for ripening and harvest. 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.

1.4. Plant habit

The cultivated cottons are annual shrubs and sub-shrubs measuring 0.6 to 2.5 m depending upon the species, cultivar and the environment. Salient morphological features of various parts of cotton plant are described as below:

Roots: There is a well developed tap root system with four rows of lateral roots. The zone of soil penetration is governed by soil structure, height of water table and the age, size and the fruiting of the plant.

Stems: The primary axis or main stem is monopodial with leaves and branches, but no flower buds. In the axil of each leaf two buds are present: a true axillary and an extra axillary or lateral bud, which may be on either side of axillary bud. Usually only one bud develops. In cotton the branching is dimorphic. Lower axillary bud produces vegetative monopodial branches. Higher on the main stem and on monopodial branches the extra axillary bud grows horizontally to give the sympodial fruiting branches. The terminal bud of the sympodial branch develops into a flower and the growth is continued by an axillary bud in the leaf axil. The weather, soil and photo period influence the number of vegetative branches developed on the plant (Purseglove 1988).

Leaves: Cotton plant has leaves spirally arranged on the monopodia with a phyllotaxy of 3/8. The leaves on the sympodia are alternately arranged in two rows. The leaves are variable in size, shape, texture and hairiness. Each leaf has extra floral nectaries on the dorsal veins and pigment glands scattered randomly on the entire surface (Purseglove 1988).

Flowers: First flower is produced after 8-10 weeks of planting. Flowers are born singly and terminally on the sympodial branch with 6-8 flowers on each fruiting branch of average length. When bractioles are first visible they are called squares and from this stage to opening of flower (Fig. 1.1 A) it takes about 21 days. Under favorable conditions flowering normally continues for two months. The time taken from flowering to opening of bolls is about 45-65 days (Purseglove 1988). Fruiting branches are sympodial and their early development begins like the vegetative branches. Flowering proceeds upwards and outwards at regular intervals. The growth habit of plants is indeterminate. Therefore the buds, flowers and bolls are present at the same time (Fig. 1.1 B) (Anthony 1991).

Fruits: Fruits called as bolls, are spherical or ovoid leathery capsules of about 4-6 cm long. Boll grows to full size in about 25 days after opening of the flower and the seed develops for a further 25 days before the boll opens. The boll splits on maturity along the carpel edges into several valves or locks, exposing linted seeds (Fig. 1.2 A & Fig. 1.2 B) (Purseglove 1988).

Seeds: The seed is pyriform and dark-brown in colour after removal of fuzz (Purseglove 1988).

1.6. Economic Importance

Cotton plant has multifarious economic uses. Seed is the most important part of the plant. Cottonseed has two principle components (i) the hull or spermoderm - the outer covering of the seed from which the cotton fiber and cotton linters arise (ii) The kernel or embryo from which oil and meal are obtained. The seed contains minor constituents such as lecithin, sterols, some vitamins of B and E group and minerals (Pandey 1998).

The oil content in the seed varies between 16-25 % of the dry seed weight depending upon the species (Lawhon *et al.* 1977). In India, the entire production of cottonseed oil is utilized for edible purpose (90-95 %), mostly for vanaspati and only small quantity is used for manufacturing of soaps (5-10 %). At present, nearly 70 % of cottonseed produced in the country is crushed for production of cottonseed oil (Pandey 1998). Other use of cottonseed oil is in production of acetoglycerides for food and cosmetics. Brominated – oils are used as wetting agents and for production of cloudy flavored soft drinks. Emulsified cottonseed oil can be used for intravenous administration to patients who require a higher calorie diet (Pandey 1998).

Cottonseed cake or meal is useful as protein rich fodder supplement (Alford *et al.* 1996). It contains about 60 % of high quality proteins and essential amino acids like lysine,

Fig. 1.1

A. Cotton flower (in bloom).

B. Sympodial fruiting branch showing flowers buds, squares and bolls.

Fig.

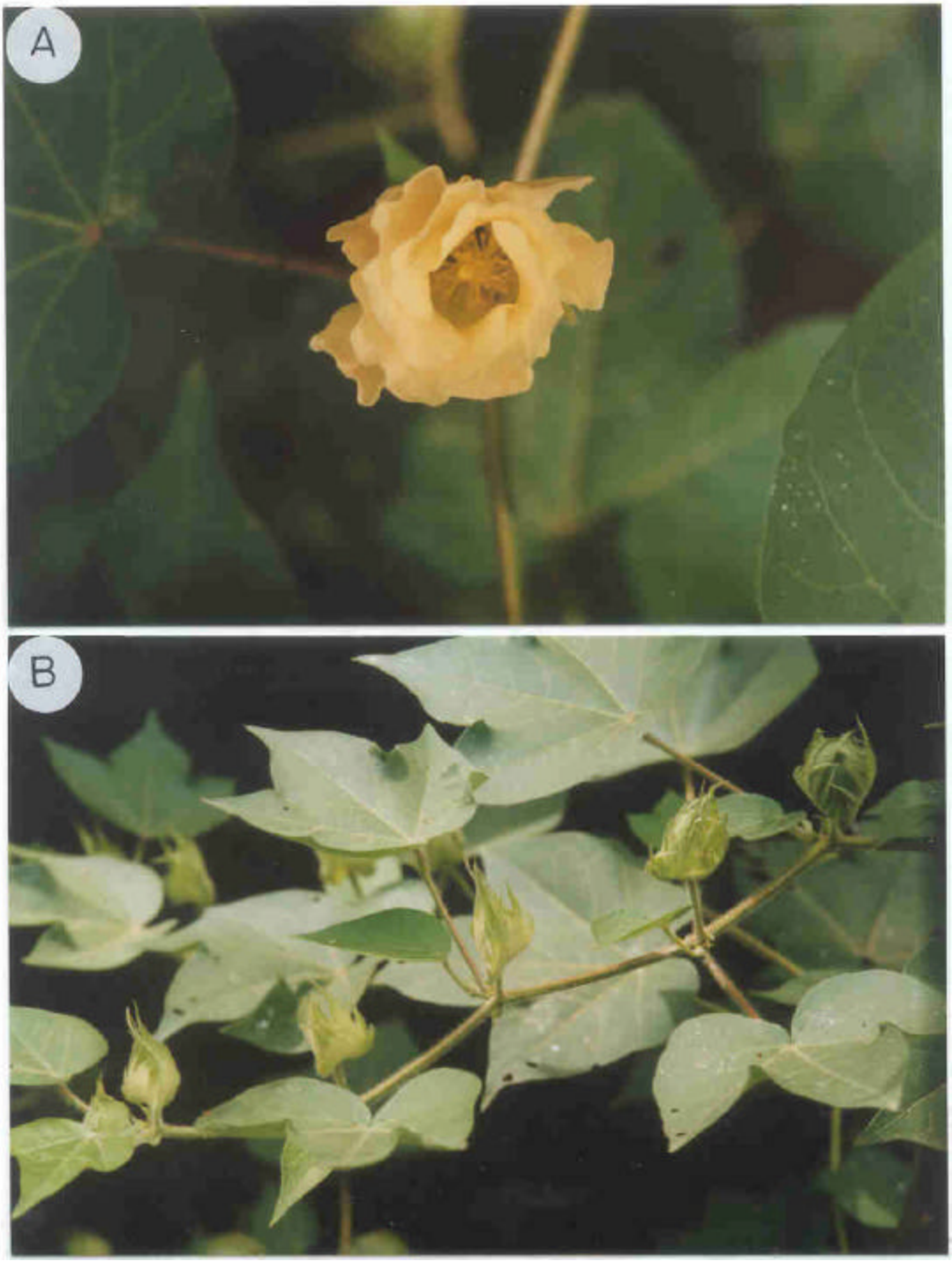


Fig. 1.2
A. Mature cotton plants with dehisced bolls.

B. The dehisced bolls exposing lint.

Fig.1.2

methionine, tryptophan, histidine, arginine etc compared to other oilseeds cakes (Beraidi and Cherry 1980). The major problem with the use of cottonseed meal or cake in the diet of non-ruminants is the presence of a number of toxic pigments such as gossypol, gossypurpurine, gossyfulvin, anthracynins and carotenes (Murray *et al.* 1993). Gossypol is a toxic yellow polyphenolic pigment located in the glands, which occurs in all parts of plant (Fryxell 1981; Khushk and Vaughan 1985). 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 anti-fertility (Hong *et al*. 1989), anti-parasitic (Eid *et al*. 1988), anti-tumor (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). These have a poor nutrient value and are 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).

Cotton linters which are short fibers hanging to the seed after ginning are used as industrial raw material for production of various types of cellulose derivatives such as cellulose nitrate, ethers, esters, acetates which are used for the production of various kinds of chemicals, plastics and fibers. Cellulose nitrate is used for production of plastics, dynamite, lacquers, finger nail polish, smokeless gunpowder and solid rocker propellant Cellulose esters and ethers are used for food castings such as bologna, sausages and frankfurters. Rayon is used for industrial fabrics. Besides these, linters are used as a source of chemical cotton for specialty paper (fine writing and bond papers, filter papers, currency, laminating papers, sanitary papers, battery separators etc) and filters, non-woven fabrics, filler aids, for films, plastics etc. Linters are also used in automotive upholstery, pads, cushions, furniture upholstery, lamp and candlewicks, twine, surgical cotton, rugs, mops, etc. (Pandey 1998; Bajaj 1998; Purseglove 1988).

1.7. Current status of cotton in India

India is the third largest global cotton producer in the world. Cotton is cultivated on 9.1 million hectare. Based on the soil-climate-variety complex, the cotton-growing tracts in India are divided into six regions (Pandey 1998), which are tabulated in **Table 1.2.** About 2.86 million tonnes of cotton lint is produced annually. The average productivity of cotton lint ranges from

320 to 600 Kg per hectare. (Sharma 2001). 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 has been given in Table 1.3.

In India, cotton is essentially grown in the *kharif*, rainy season and is treated as a perennial crop. Only 10 % of the cotton crop is irrigated. Major portion of the irrigated crop is in the northern *hirsutum-arboreum* regions. Optimum sowing time in this region is end of April. In the Central *arboreum* region, cotton is grown in *kharif* season. Most of the crop in this region is rainfed and is sown in May/June. The Southern *hirsutum-arboreum* region represents about 5% of total area of cotton and 8 % of country's total production. The harvesting season for the irrigated crop is January to March and for unirrigated crop upto June-July. The central *herbaceum-arboreum* region constitutes 21.5 % of total area under cultivation and 14.2 % of total production. The crop is sown from May to July and is harvested from October to January. In the Western *herbaceum* region, cotton is sown in June/August and harvested between February and April. In this region, cotton crop is a long season crop and remains in field for about nine months. In the Eastern region the crop is sown in April to June. The *rabi* cotton comes to harvest in February. In hilly areas of Assam, Manipur and Tripura, the only cotton of commercial importance called comillas is cultivated (Pandey 1998).

Table 1.2: Cotton growing regions in India.

Regions	States / Locations	Major cotton species grown
Northern *hirsutum - arboreum* region	Punjab, Rajasthan, Uttar Pradesh	*G. hirsutum, G. arboreum*
Central *arboreum* region	Gujarat, Madhya Pradesh, Southern Rajastan, South Saurastra, Khandesh, Vidharbha and Marathwada regions of Maharashtra, and Adilabad distric of Andhra Pradesh	*G. hirsutum, G. arboreum*
Central *herbaceum - arboreum* region	Andhra Pradesh and Karnataka	*G. herbaceum,* *G .arboreum*
Southern *hirsutum - arboreum* region	Tamilnadu and Kerla	*G. herbaceum, G. hirsutum, G. arboreum, G. barbadense*
Western *herbaceum* region	Most of Gujarat and kumpta cotton tract of Mumbai-Karnataka	*G. herbaceum*
Eastern region	Orissa, Bihar, West Bangal Assam, Tripura and Manipur	*G. arboreum*

(Source: Pandey 1998)

Table 1.3: State wise cotton production in India (1998 -1999).

States	Area under cultivation (Million hectares)	Production (Million tonnes)	Average yield (kg / ha)
Maharashtra	3.20	2.62	139
Gujarat	1.61	3.94	416
Punjab	0.56	0.60	180
Andhra Pradesh	1.28	1.49	198
Haryana	0.58	0.87	255
Rajasthan	0.64	0.87	230
Karnataka	0.61	0.86	239
Madhya Pradesh	0.50	0.43	145
Tamilnadu	0.24	0.43	301
Others	0.07	0.07	-
TOTAL INDIA	**9.29**	**12.8**	**223**

(Source: Agricultural Statistics at a glance 2000, Department of Agriculture and Cooperation, Government of India)

In India stable varieties of all four cultivated species of cotton i.e. *G. 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* are grown (Basu 1995). The 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 – 26 mm) and extra-long staple (27 mm and above), 45 % to the lower medium (20mm – 21.5mm) + superior medium (22 mm – 24 mm) and 5 % to the short staple (19mm and below) categories. Hybrids contribute almost 45 % of the total lint production (Basu 1995). At Central Institute for Cotton Research (C.I.C.R), Nagpur, India, a National Gene Bank of cotton genetic resources holds nearly 9000 accessions including four cultivated species, 25 wild species and a large number of perennial cottons (Basu 1995). Six important cultivars have been chosen for the present study

and details of these cultivars have been given in Table 1.4. India's import and export of cotton lint in the last five years have been given in Table 1.5.

Table 1.4: Details of the six Indian cultivars of cotton (*G. hirsutum* L) used in the present study.

Characters/ Source etc.	Cultivars		
	NHH-44	**DCH-32**	**DHY-286**
Type of hybrid	Intra hirsutum	Inter specific	Intra hirsutum
Place where developed	MAU, Nanded, MS, India	UAS, Dharwad, KN, India	Dr.PDKV, Akola, MS, India
Year of release	1985	1983	1978
Area were cultivated	AP, MS, India	KN, A.P, TN, GJ, India	MS, India
Plant habit			
Growth	Annual, Erect	Annual, Erect	Annual, Erect
Height	150 cm	150 cm	120 cm
Boll size	Roundish medium	Large roundish	Medium
No. of locules	4	3-4	4
Crop duration (no. of days)	165 days	180 days	190 days
Fiber length	25 mm	33 mm	27 mm
Staple class	Medium staple	Extra long staple	Medium staple
Resistance	Jassid Aphid	-	Jassid
Yield(q/ha) Irrigated→ Rainfed →	 30 – 35 q/ha 8 – 10 q/h	 35 – 40 q/ha -	 - 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,	CICR, RS, Coimbatore, India	CICR, RS, Coimbatore, India
Year of release	1993	1992	1982
Area/s where cultivated	MS, India	MS & TN, India	MS & TN, India
Plant habit			
Growth	Annual, erect	Annual, erect	Annual, erect
Boll size	Medium	Big	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 1.5: India's Import and Export of cotton lint.

Year	Import		Export	
	Quantity in (Metric tonnes)	Value (1000 $)	Quantity (Metric tonnes)	Value (1000 $)
1999	55,685	90,306	20,000	22,000
1998	55,685	90,306	32,211	38,171
1997	8,824	21,316	139,520	197,549
1996	2,860	8,795	253,414	410,751
1995	69,451	161,513	26,676	50,963

(**Source:** FAOSTAT Database)

1.6.1. Desi cottons

Two diploid cotton species *G. arboreum* L. and *G. herbaceum* L. are together known as "Desi" cottons ("Desi" literally means native). India is considered to be the birth place of these two species which 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

Colored cottons (naturally pigmented 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 (Fig. 1.3). 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 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 tackled by cultivation of colored cottons in isolated areas under strict legislation (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).

1.8. Factors affecting cotton production

1.8.1. Abiotic Stresses

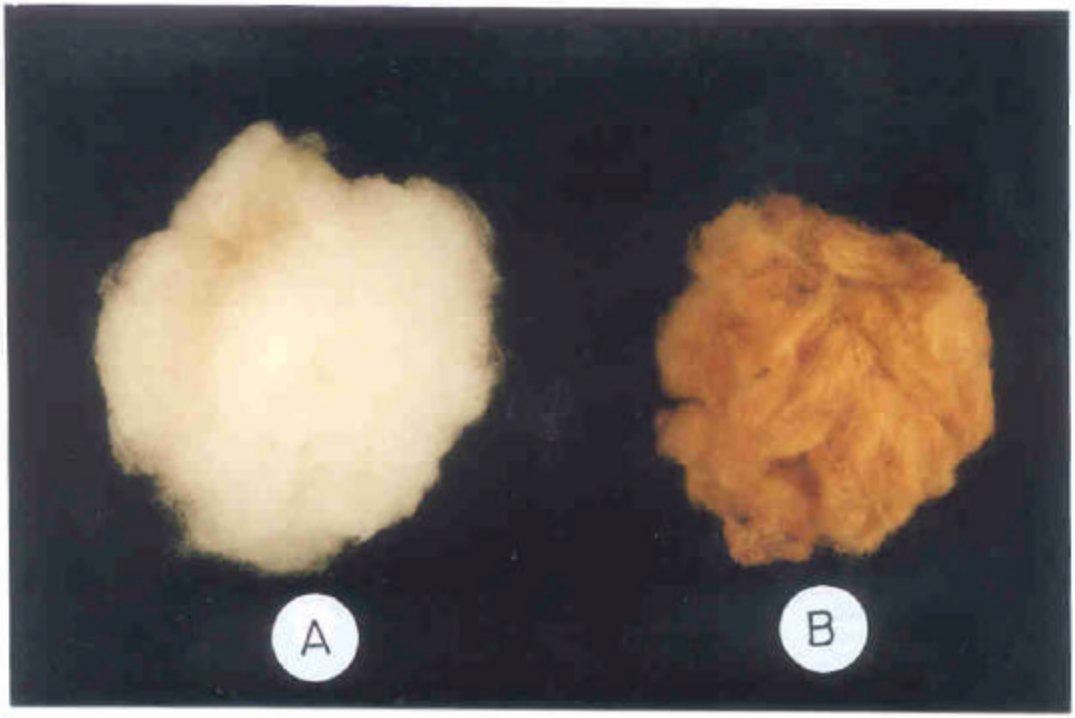
Abiotic stresses such as salinity and drought are the main constraints to crop productivity. These factors have negative effects on the cotton yield (John 1997). Salinity induces nutritional imbalances (Martinez & Lauchli 1994) and affects the cotton growth, yield (Nawar *et al*. 1994) and fiber quality (Razzouk & Whittington 1991). Tolerance to salinity varies from germination to vegetative stages; cultivar that appears fairly tolerant to salinity during germination can be sensitive during the vegetative stage (Lauchli *et al.* 1981). Also it varies with the genotypes both at vegetative as well as reproductive stages (Leidi 1994). Under water

stress, cotton plants reduce root and shoot growth differentially, increasing the root/ shoot ratio (Malik *et al.* 1979; Ball *et al.* 1994).

Fig.1.3

Naturally pigmented brown cotton (**B**), white cotton (**A**).

Fig.1.3



1.8.2. Biotic stresses

1.8.2.1. Insect pests

Due to insect pest and diseases, there are tremendous losses in cotton production and fiber quality. About 162 species of insects are known which attack the cotton plant at various stages of growth (Sharma 2001). Cotton growers use almost half of the insecticides applied to crops in the United States (Adkisson *et al*. 1982). Some of the most important insects causing considerable damage to the crop are white fly (*Bemisia tabaci*), pink bollworm (*Pectinophora gossypiella*), boll weevil (*Anthonomous grandis*), American bollworm (*Heliothis virescens*) etc. These bollworms which are the caterpillars of several species of moths feed in the boll (Fig. 1.4) damaging lint and seeds. These cause considerable reduction in yield and quality. In spite of the use of chemical pesticides to control cotton insects, yield losses occur. The combined cost of chemicals and yield losses in the US alone is up to 650 million US $ per year (Perlak *et al.* 1990). Over 200 million US $ are spent annually for crop protection against insects (Jenkins *et al.* 1991). In the United States, three fourth of the damage to cotton crop is caused by the boll weevil and the cotton bollworms (Pendergrass 1989). Boll weevil attacks the young squares, bolls and terminal buds. Leaf, stem and bud sucking bugs also cause considerable damage to cotton (Purseglove 1988).

In India, the important insect pests which cause considerable losses in cotton production are sucking pests - jassids, aphids; insects – whitefly, bollworms viz American bollworm, pink bollworm, spotted bollworm. Beside these, leaf eating caterpillars (*Spodoptera litura*) and stem weevil (*Pempherulus affinis*) also damage the crop in some areas in south of India. Aphids, jassids and thrips in isolated areas 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

Fig. 1.4

Cotton boll infected with the bollworm (*Heliothis*).

Fig.1.4

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 suicides by more than 500 cotton farmers in Andhra Pradesh, Karnataka, Maharashtra and Punjab who failed to return the loans taken from moneylenders for purchase of pesticides to save their crop (Sharma 2001). Out of total estimated Rupees 28 billion (US$ 620 million) of chemical pesticides used in crop protection in the country, Rupees 16 billion (US $ 344 million) is utilized on cotton alone (Sharma 2001). In spite of this huge expenditure, insect menace still remains a major concern in cotton crop production in India. A list of major insect pests causing damage to cotton crop is given in Table 1.6.

Table 1.6: Insect pests of cotton crop.

Insect pests	**Scientific names**
Boll weevil	*Anthonomous grandis*
Spotted bollworm	*Earis spp.*
American bollworm	*Heliothis spp.*
Pink boll worm	*Pectinophora gossypiella*
Leaf worm	*Alabama argillacea*
Aphid	*Aphis gossypii*
Thrips	*Thrips tabaci*
Flea hopper	*Psallus seriatus*
Tarnished and rapid plant bugs Jassid	*Lygus hesperus* *Amrasca biguttuals biguttula*
Whitefly	*Bemisia tabaci*
Cabbage looper	*Trichoplusiani*

1.8.2.2. Major diseases

The major bacterial and viral diseases in cotton 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 spread 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 1.7.

Table 1.7: Some common diseases affecting cotton.

Type of Causal organism	Disease	Name of the causal organism
Bacteria	Bacterial blight	*Xanthomonas malvacearum*
Fungus	Ascochyt blight	*Ascochyta gossypii*
	Anthracnose	*Glomerella gossypii*
	Fusarium wilt	*Fusarium oxysporum f. vasinfectum*
	Cotton rust	*Puccinia cabata*
	Verticillium wilt	*Verticillium alboatrum*
Nematode	Root rot	*Phymatotrichum omnivorum*
	Root knot nematode	*Meloidogyne incognita*
	Rhizoctonia root rot	*Rhizoctonia solani*
Virus	Leaf curl Virus	*Bemisia tabaci* (vector)

1.9. Improvement of cotton

A typical cotton improvement program aims at one or more of the following objectives: increasing the production of lint fiber, upgrading the fiber quality, early maturing types, gossypol free seed and development of resistance to various insects, diseases and nematodes (Bajaj 1998).

1.9.1. Conventional methods

Cotton is generally a self-pollinated crop. The amount of cross pollination depends to a large extent on the insect population present in the field. 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 due to cultivation of 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 contributing more than 45% of the total lint production in the country (Basu 1995).

1.5. Haploid cotton plants

Haploid plants some times are naturally found in cotton as a twin seedling once in about every 50,000 to 100,000 plants. Haploids are identified by a thin stem, short internodes, small leaves and floral parts and failure to shed pollen. They usually lack seeds and bolls and may be taller than the normal plants in the field. These may be used for the production of doubled haploids by repeated application of 0.2 % colchicine solution. Doubled haploids hold considerable interest since they are extremely uniform, comparable to pure lines and can be maintained by controlled self-pollination (Mayer & Justus 1961). Doubled haploids could contribute towards a more effective utilization of intraspecific as well as interspecific heterosis in cotton (Bajaj 1998).

1.9.2. Need for Non conventional methods

Though introduction of hybrid varieties developed by conventional breeding methods has lead to a spectacular improvement in yield and fibre traits, this approach has limitation as it is laborious, time consuming and involves high costs due to manual emasculation and pollination (Srinivasan *et al.* 1972; Davis 1978).

Several wild species of *Gossypium* though are short fibered or lintless possess other desirable traits as mentioned in Table. 1.8. Successful transfer of these traits to cultivated tetraploid species has not been achieved due to various incompatibility barriers such as abortion of embryo and/or degeneration of endosperm (Weaver 1958; Pundir 1972). Thus, there is a strong need to develop non conventional biotechnological methods that can ensure hybridization between distinctly related species. The introduction of genetic variability through somaclonal variation, genetic transformation also needs to be incorporated in cotton breeding programs. The potential applications of some of the target genes are mentioned in Table 1.9.

Table 1.8: Wild species of cotton possessing useful traits.

Species	Useful traits
G. somalense (Gurke) J.B.Hutch.	Resistance to bollworm
G. armourianum Kearn.	Resistance to bollworm, increased number of ovules per loculus
G. thurberi Tod.	Resistance to bollworms, high fiber strength
G. raimondii Ulbr.	Resistance to jassids and tolerant to drought, high density of seed hairs
G. harkensii Brendeg.	Resistance to drought and spider mites, source of cytoplasmic male sterility
G. anomalum Wawrex Wawra & Peyr	Lint quality, resistance to jassid
G. aridum (Rose & Standl.) Skov.	Tolerance to drought
G. stocksii Mast.in Hook.	Resistance to drought
G. tomentosum Nutt.ex Seem.	Resistance to drought and jassids, lint quality
G. bickii Prokh.	Gossypol – free seeds

(Source: Bajaj 1998)

Table 1.9: The potential application of some target genes for genetic engineering in cotton.

Target gene/s	Potential application
Insecticidal gene/s	
Bacillus thuringgiensis toxin	Control of *Helizoverpa zea, Heliothis virescens, Pectinophora gossypiella*
Protease inhibitor, lectins	Feeding deterrents
Venoms, neuropeptides	Kill or paralyze feeding pests
Herbicidal gene/s	
5-Enolpyruvylshikimic acid 3-phosphate	Glyphosphate tolerance
Nitrilase	Bromoxynil tolerance
Acetolactate synthase	Sulfonylurea tolerance
2,4-Dichlorophenoxyacetate monooxygenase	2,4-D tolerance
Phosphinotricin acetyltransferase	Bialaphos tolerance
Environmental stress-resistance-tolerance gene/s	
Superoxide dismutase	Free radicle quenching
Thermal and water stress-tolerance gene	Heat, cold and drought tolerance
Fiber-modification gene/s	
Cotton genes	Modification of existing fiber properties
Other plant genes (extensins, peroxidase)	Modification of existing properties
Bacterial genes (e.g., hormone genes)	Modification of existing properties
Genes for hybrids	
Pollen-specific antisense genes	Production of mail sterile plants
Cytotoxic genes (e.g., Rnase)	Production of proprietary seeds

(Source: Bajaj 1998)

1.10. Biotechnological approaches

In the last two decades, several reports have been published on various aspects of biotechnological studies in cotton. These include callus initiation, somatic embryogenesis, organogenesis, protoplast culture, inter-specific hybridization through embryo or ovule culture, somaclonal variation and plant transformation etc. These reports have been summarized in the following sections.

1.10.1. Callus initiation

The available reports on establishment of callus cultures are listed in table 1.10. In these reports, the main objective of callus initiation was to achieve plant regeneration via organogenesis or embryogenesis, isolation of protoplasts and production of somatic hybrids. Callus induction was achieved from various explants such as anther, hypocotyl sections, mesocotyl, cotyledon and leaf pieces, petiole and stem sections etc. However, differentiation of callus in all these reports could not be achieved.

Table 1.8: Studies on establishment of callus cultures in cotton species.

Species	Explant/s used	Reference
G. hirsutum L.	Mesocotyl	Schenk & Hilderbrandt (1972)
G. hirsutum L.	Leaf	Davis *et al.* (1974)
G. hirsutum,L.;*G. arboreum* L.	Hypocotyl and Stem	Rani & Bhojwani (1976)
G. barbadense L. *G. davidsonii* Kell.	Cotyledon	Katterman *et al.* (1977)
G. anomalum Wawr. Ex Wawr. & Peyr.; *G. arboreum* L.; *G. armourianum* Kearn.; *G. hirsutum*,L.; *G. klotzschianum* Anderss *G. raimondii* Ulbr.	Hypocotyl	Price *et al.* (1977)
G. arboreum L. *G. hirsutum* L.	Hypocotyl	Smith *et al.* (1977)
G. hirsutum, L. *G. arboreum* L.	Hypocotyl	Bajaj & Gill (1985)
G. arboreum L. *G. hirsutum* L.	Hypocotyl	Zimmerman & Robacker (1988)

G. arboreum L.	Anther	Bajaj & Gill (1989)

1.10.2. Somatic embryogenesis

The ability of regeneration of plants from the callus or cell suspension via organogenesis or somatic embryogenesis is a basic pre-requisite for the application of cell culture techniques to crop improvement. Price and Smith (1979) were the first to report the somatic embryogenesis in wild species of cotton (*G. klotzschianum*). However, somatic embryos could not develop into plantlets. Regeneration of complete plants *via* somatic embryogenesis in specific callus lines of cotton was first achieved by Davidonis & Hamilton (1983). However, the method described by them has limitation due to prolong incubation period (two years) of callus for induction of proembryoids with low frequency of embryo formation. Shoemaker *et al*. (1986) evaluated 17 cultivars of cotton (*G. hirsutum* L.) and found two cultivars Coker 201 and Coker 315 as embryogenic. The procedure described by Shoemaker et. al. is simple and rapid. In their report, less than 40% of the somatic embryos underwent normal germination producing roots and shoots upon transfer to auxin free medium with reduced sugar level. Gawal *et al.* (1986) reported somatic embryogenesis from the callus cultures of mature leaf and petiole explants. Trolinder & Goodin (1987, 1988 a, b), Finer (1988) could achieve the regeneration of cotton plants from embryogenic suspension cultures. They concluded that induction of somatic embryogenesis in cotton is genotype dependent. Plant regeneration in Indian cultivars 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 embryos (14.28 per explant).

Table 1.10: Studies on somatic embryogenesis in cotton.

Genotype	Explant Used	Mode of Regeneration	Reference
G.klotzschianum Anderss	Hypocotyl	Callus SE	Price & Smith (1979)
G. hirsutum L.	Cotyledon	Callus PE Plantlet	Davidonis & Hamilton (1983)
G.klotzschianum Anderss.	Stem, Petiole, Leaf disc	Callus SE	Finer & Smith (1984)
G. hirsutum L.	Hypocotyl, Immature embryo	Callus SE Plantlet	Rangan *et al.* (1984)
G. hirsutum L. G.hirsutum L.	Hypocotyl Leaf disc, petiole	Callus SE Plantlet Callus SE Plantlet	Shoemaker *et al.* (1986) Gawel *et al.* (1986)
G.hirsutum L.	Hypocotyl	Callus SE Plantlet	Umbeck *et al.* (1987)
G.klotzschianum Anderss.	Hypocotyl	Callus Susp.cult. SE Plant let	Finer *et al.* (1987)
G.hirsutum L. *G. hirsutum* L.	Hypocotyl Hypocotyl	Callus SE Plantlet Callus SE Plantlet	Tolinder & Goodin (1987) Tolinder & Goodin (1988 a)
G. hirsutum L.	Hypocotyl	Callus SE Plantlet	Tolinder & Goodin (1988 b)
G. hirsutum L.	Cotyledon	Callus Susp.cult. SE Plant let	Finer (1988)
G. hirsutum L. *G. barbadense* L. *G. arboreum* L.	Hypocotyl	Callus SE	Trolinder & Xhixian (1989)
G. hirsutum L.	Petiole	Callus SE	Gawel & Robacker (1990 a)
G. hirsutum L.	Hypocotyl	Callus SE Plantlet	Voo *et al.* (1991)
G. hirsutum L.	Cotyledon, Hypocotyl	Callus SE Plantlet	Firoozabady & De Boer (1993)
G. hirsutum L.	Hypocotyl	Callus SE Plantlet	Kumar & Pental (1998 a)
G. hirsutum L.	Hypocotyl	Callus SE Plantlet	Kumar & Pental (1998 b)
G. hirsutum L.	Cotyledon, Hypocotyl	Callus SE Plantlet	Zhang *et al.*(2000 a)

SE: Somatic embryo; Susp. Cult.: Suspension culture

1.10.3. Organogenesis

An alternative to somatic embryogenesis approach is plant regeneration from explants having pre-existing meristems. Apical meristem culture of cotton was first reported by Chappel & Mauney (1967). However, they could not obtain complete plants. In their studies, although new leaf primordia could be initiated, root system failed to develop. Bajaj & Gill (1986) obtained 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 of *in vitro* grown seedlings was first reported by Agrawal *et al*. (1997). After that several reports on plant regeneration via pre-existing meristems have been published. These have been listed in Table 1.11.

Table 1.11: Studies on plant regeneration in cotton from explants having pre-existing meristems.

Species	Explant used	Response	Reference
G. arboreum L. *G. hirsutum* L.	Apical meristem, shoot tip	Adventitious buds & multiple shoots	Bajaj & Gill (1986)
G. hirsutum L. *G. barbadense* L.	Shoot apex	Single shoot	Gould *et al.* (1991)
G hirsutum L.	Cotyledonary node	Multiple shoots	Agrawal *et al.* (1997)
G. hirsutum L. *G. arboreum* L.	Shoot apex with 2 cotyledons, shoot apex with one cotyledon, shoot apex without cotyledons,	Multiple shoots	Gupta *et al.* (1997)
G. hirsutum L.	Shoot tip	Single shoot	Sayeed *et al.* (1997)
G hirsutum L.	Split embryo axis	Single shoot	Agrawal *et al.* (1998)
G. hirsutum L.	Shoot apices, secondary leaf nodes, primary leaf nodes cotyledonary nodes	Multiple shoots	Hemphill *et al.* (1998)
G. hirsutum L.	Caulinar apex	Multiple shoots	Morre *et al.* (1998)

G. hirsutum L.	Shoot apex	Single shoot	Zapata *et al.* (1999 b)
G. hirsutum L. *G. arboreum* L.	Cotyledonary node, split cotyledonary node, shoot tip, petiole base	Multiple shoots	Hazra *et al.* (2000)

1.10.4. Embryo Rescue

Embryo rescue technique in cotton has been used for the production of inter-specific hybrids involving various wild and cultivated species, both diploid and tetraploids. (Bajaj & Gill 1998). Successful 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 been reported. In all these crosses, 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.

1.10.5. *In vitro* fertilization

In vitro fertilization and pollination techniques have been used in producing hybrids in incompatible crosses (Bajaj 1979). This technique involves growing unpollinated flowers, ovaries and ovules on medium and then sprinkling pollen over them or on the cut end of the style. Rafaat *et al*. (1984) fertilized *G. hirsutum in vitro* with pollen of *G. barbadense* and obtained hybrids of cotton. Liu *et al*. (1992) successfully produced hybrids between *G. hirsutum* L. and *G. arboreum* L. by this technique. They studied various factors such as culture medium, temperature, relative humidity and incubation conditions such as stationary vs. shaking etc. Although *in vitro* fertilization resulted in formation of plants and seeds, these were sterile.

1.10.6. Ovule Culture

Cotton ovule culture is a valuable technique to circumvent the problems encountered in hybridization between diploid and tetraploid species. The technique has other applications such as (1) understanding the development of fiber *in vitro* (Trolinder *et al*. 1987; Seagull 1990), (2) study of peroxidases secreted in to the culture medium by ovules (Mellon 1991), (3) as a model system for investigation of interactions between cotton and toxigenic fungus *Aspergillis flavus* (Mellon 1992). Successful development of ovule to whole plant seems to depend on factors like phyto-hormone and parental germplasm. Full potential of ovule culture for improvement of commercial cotton germplasm remains yet to be tapped. Reviews on cotton

ovule culture describing methods, applications and successful reports have been published (Stewart 1991; Beasley 1992).

1.10.7. Protoplast culture

Isolated protoplasts are considered to be ideal explants for genetic transformation because of the freely accessible plasmalemma and non-involvement of biological vector in the transformation process (Peeters *et al*. 1994). Several studies have been conducted on the isolation and culture of cotton protoplasts. Protoplasts 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 to plant. Successful plant regeneration from protoplasts derived from embryogenic cell suspensions was achieved by Chen *et al*. (1989); She *et al*. (1993); Peeters *et al*. (1994). Several other reports on protoplast culture in cotton have been listed in Table 1.12.

Table 1.12: Studies on protoplasts in cotton.

Species	Donor tissue	Response	Reference
G. hirsutum L.	Hypocotyl callus	Macro colonies	Bhojwani *et al.* (1977)
G.klotzschianum Anderss.	Hypocotyl callus	Macro colonies	Finer & Smith (1982)
G. hirsutum L.	Anther callus	Callus	Thomas & Katterman (1984)
G. hirsutumi L *G. barbadense* L.	Cotyledon	Micro colonies	Firoozabady & DeBoer (1986)
G. hirsutum L.	Stem callus	Callus	Saka *et al.* (1987)
G. hirsutum L.	Embryogenic suspension	Micro colonies, plant	Chen *et al.* (1989) She *et al.* (1993)
G. hirsutum L.	Embryogenic suspension	Fertile plant	Peeters *et al.* (1994)

1.10.8. Somaclonal Variations

The genetic variability in a population is a key factor in any crop improvement program. 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 collectively referred to as "somaclonal variations" are a rich source of genetic variability. Bajaj & Gill (1985) have reported chromosomal changes in cotton cell cultures. The plants showing tolerance to high temperature and cell lines tolerant to salt were obtained. Stelly *et al*. (1989) observed that somaclonal plants regenerated from callus cultures of *G. hirsutum* L. extremely varied in phenotypic characters. Trolinder and Goodin (1988b) observed that among somaclones of cotton, 84.6 % plants were infertile. Trolinder & Xaiomin (1991) regenerated high temperature resistant cotton plants from selected somaclones of *G. hirsutum*. Yet in an another study, Reynaerts and De Sonville (1994) have reported *in vitro* induction of mail sterility in cotton.

1.10.9. Genetic transformation of cotton *via Agrobacterium tumefaciens*

Firoozabady *et al*. (1987) and Umbeck *et al*. (1987) were the first to report genetic transformation in cotton *via Agrobacterium tumifacience* mediated transformation. Perlak *et al.* (1990) were the first to report expression of *Bacillus thuringiensis* var. kurstaki (*cryIA (b)* and *cryIA(c)* genes, conferring resistance to insects in cotton. In an another report, 2,4-D resistant trait in cotton could be engineered by transferring the 2,4–D mono oxygenase gene *tdf*A from *Alcaligenes eutrophus* (Bayely *et al*. 1992; and Lyon *et al.* 1993). The expression of Protease inhibitor gene in cotton plant has also been reported (Thomas *et al.* 1995) following the *Agrobacteirum* meditaed transformation. Herbicide resistant transgenic cotton carrying mutant forms of a native acetohydroxyacid synthase (AHAS) gene have been obtained (Rajasekaran *et al.* 1996 b). Recently, 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.

1.10.10. Genetic transformation by Particle bombardment method

Presently, several reports are available on transformation of cotton tissues by particle bombardment method. This method of gene transfer was developed by Klein *et al.* (1987). Finer and McMullen (1990) bombarded embryogenic cell suspension of cotton variety Coker 310 with hygromycin gene and obtained hygromycin resistant plants. McCabe & Martinell (1993) described the protocol for a variety independent transformation of cotton by particle bombardment with excised meristems from embryo axes derived from seed. In similar studies, Chlan *et al.* (1995) and Keller *et al.* (1997) also used embryo axis explants as targets for

bombardment to develop transgenics in cotton. Rajasekaran *et al.* (2000) bombarded embryogenic cell suspensions with particles coated with chimeric genes and developed transgenic plants via somatic embryogenesis. Reports on cotton transformation both by *Agrobacterium tumefaciens* and particle bombardment mediated techniques have been listed in Table 1.13.

Table 1.13: Studies on genetic Transformation in cotton by *Agrobacterium* and particle bombardment methods.

Species	Explant Used	Method Used	Response	Reference
G. hirsutum L.	Cotyledon	*Agrobacterium tumefaiciens*	Somatic embryo	Firoozabady *et al*. (1987)
G. hirsutum L.	Hypocotyl	*Agrobacterium tumefaiciens*	Somatic embryo	Umbeck *et al*. (1987)
G. hirsutum L.	Embryogenic cell suspension	Particle bombardment	Somatic embryo	Finer & McMullen (1990)
G. hirsutum L.	Hypocotyl	*Agrobacterium tumefaiciens*	Somatic embryo	Perlak *et al.* (1990)
G. hirsutum L.	Hypocotyl	*Agrobacterium tumefaiciens*	Somatic embryo	Bayley *et al.* (1992)
G. hirsutum L.	Meristem	Particle bombardment	Single shoot	McCabe & Martinell (1993)
G. hirsutum L.	Cotyledon	*Agrobacterium tumefaiciens*	Somatic embryo	Thomas *et al*. (1995)
G. hirsutum L.	Meristem	Particle bombardment	Single shoot	Chlan *et al*. (1995)
G. hirsutum L.	Embryogenicc ell suspension	*Agrobacterium tumefaiciens* & Particle bombardment	Somatic embryo	Rajasekaran *et al.* (1996 b)
G. hirsutum L. *G.barbadense* L.	Meristem	Particle bombardment	Single shoot	Keller *et al*. (1997)
G. hirsutum L.	Shoot apex	*Agrobacterium tumefaiciens*	Single shoot	Zapata *et al.* (1999 a)
G. hirsutum L.	Embryogenic cell suspension	Particle bombardment	Somatic embryo	Rajasekaran *et al.* (2000)

1.10.11. Genetic engineering of cotton fiber

Cotton fiber, a source of pure cellulose is highly valued for the manufacture of a large percentage of world's textiles. Cotton fiber is a differentiated single epidermal cell of the outer integument of the ovule. It undergoes 1000-3000 fold increase in length during final stages of differentiation (Trolinder *et al*. 1987; Bajaj 1998). Some of the specific modifications of fiber useful for various applications are superior strength, length, dye binding, dimensional stability and thermal adaptability. Recombinant DNA technology and improved transformation methods have application in production of new and improved fibers that could compete with synthetic ones (John 1994, 1996). Production of thermoplasitc polymer polyhydroxybutyrate (PHB) in cotton fibers was obtained through particle bombardment mediated plant transformation (Rinehart 1996). John and Keller (1996) genetically engineered cotton fiber to produce an aliphatic polyester compound polyhydroxyalkanoate (PHA).

1.11. Transgenic cotton – present status

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

Cotton is among the first few transgenic crops that have been commercialized successfully. The 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. After several year's field trials, 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 area (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). China and Australia are other countries where Bt cottons have been planted in field. In 1998, over 100,000 hectares of Bt cotton were planted in China. (Zhang *et al.* 2000 b).

1.11.2. Herbicide resistance

For the last fifty years, 2,4-dichlorophenoxyacetic acid (2,4-D) has been commonly used as a herbicide to control broadleaf weeds. However, due to its volatility, it causes severe damage to nearby crops. In two independent studies, transgenic cotton resistant to 2,4-D has

been developed (Bayley *et al*. 1992 and 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, sorghum and pasture crops. 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) transformed Acala and Coker varieties resistant to imidazolinone herbicides. It was observed that transgenic progeny was resistant to imidazolinone herbicides at five times the field application level. Other cotton transgenics like "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 at the verge of release soon (Zhang *et al.* 2000 b).

Thus, USA has taken a lead followed by China and Australia in development and commercialization of several cotton transgenics. 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).

1.12. Aims of the thesis

From the literature survey it becomes obvious that cotton in general and Indian cotton cultivars in particular are recalcitrant to *in vitro* culture. A majority of reports published are on a few Coker varieties, which are not cultivated in India. In the absence of suitable plant regeneration protocols via organogenesis/somatic embryogenesis for Indian cultivars of cotton, regeneration through explants with pre-existing meristems is an alternative option for rapid clonal propagation and genetic transformation.

The present study was undertaken with the objective of direct plant regeneration from the explants consisting of pre-existing meristems of Indian cultivars of cotton and its application in the genetic transformation by particle bombardment method. Thus the aims of the present study are:

1. to develop a simple and rapid method of clonal propagation from the explants derived from mature zygotic seeds.
2. to develop a highly reproducible and efficient regeneration system from explants with the pre-existing meristems derived from *in vitro* grown seedlings.
3. to develop a method of clonal propagation in cotton using nodal segments from field grown mature plants,
4. to optimize different parameters of particle bombardment method for transient gene expression in embryo axis explants.

Chapter 2

Materials and Methods (General)

The techniques routinely followed during the entire course of present investigations particularly in plant tissue culture work have been described in this chapter. Surface sterilization method used for the nodal segments of the field grown plants has been described in chapter 5. The methods used in genetic transformation study by particle bombardment have been described in chapter 6 of the thesis.

2.1. Plasticware

Pre-sterilized plastic petridishes of 35 mm, 55 mm and 85 mm diameter, sterile, disposable filter sterilization units having filters of pore size 22 μm procured from "Laxbro", India were used during the experiments. Eppendorf tubes (1.5 ml and 2 ml capacity), micro-tips (0-200 μl and 200-1000 μl capacity) obtained from "Laxbro" and "Tarsons", India and wide bore micro-tips (0-200 μl) procured from "Sigma", USA were used as other plasticware.

2.2. Glassware

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

2.2.1. Preparation of Glassware

Glassware used for all the experiments was initially cleaned by boiling it in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Then they were immersed in 30% nitric acid solution for 30 min followed by repeated washing in tap water. Thereafter they were rinsed with distilled water and dried at room temperature or in an oven at 200 °C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd, Andhra Pradesh, India). Pipettes and petri dishes were wrapped in brown paper and then sterilized in an autoclavable polypropylene bag. Ordinary filter paper pieces (approx. 10 x 20 cm) were autoclaved in a polypropylene bag. These were used for dissection and for transfer of explants in a laminar air flow cabinet. Autoclaving of the glassware and above items was carried out at 121°C, 15 lb psi for 1 h.

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 experiments 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, fructose, maltose, were obtained from "Qualigens" and agar-agar from "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO®" laboratories, USA.

2.4. Preparation of culture media

Glass distilled water was used for the preparation of culture media. After addition of all macro- and micro-nutrients, vitamins, growth regulators and other necessary carbohydrate source like sucrose or glucose or fructose or maltose, the pH of the media was adjusted to 5.8 before autoclaving using 0.1N NaOH or HCl. When activated charcoal (Sarabhai Chemicals, India) was added to the medium, the pH was adjusted to 6.0 before autoclaving of the medium. The volume of the medium 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. These were added to autoclaved medium before dispensing. The compositions of Murashige and Skoog's (MS) macro-, micro elements, vitamins and Gamborg's (B5) vitamins used in the present study are given in Table 2.1, 2.2 and 2.3 respectively.

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

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

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

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

Table 2.3: Composition of organics in MS (Murashige & Skoog 1962) 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 and DHY-286 were obtained in the month of June - July from Maharashtra State Seed Corporation (MSSC), Akola, Maharashtra, India. The seeds of cultivar CNH-36 were obtained from Central Institute for Cotton Research (CICR), Nagpur, India. The seeds were stored in sealed containers at room temperature and used throughout the year.

2.6. Preparation of plant material

2.6.1. Surface sterilization of seeds

The delinted seeds (Fig.2.1A) of the above mentioned cultivars were washed with 1 % (vol/vol) Labklin soap solution (S.D.Fine Chem, India) for 5 minutes. 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 10 minutes. Savlon was removed by 2-3 rinses of seeds with running tap water and twice with glass distilled water (45-50 secs for each wash). The seeds were surface sterilized with 0.1 % (wt/vol) mercuric chloride (Qualigens, India) solution for 10 minutes followed by 4-5 rinses of one min duration each with sterile glass- distilled water. After that, the seeds were aseptically transferred to a 250 ml capacity flask containing charcoal suspension (0.25 % charcoal in glass distilled water) and were allowed to soak for 4 hours. These were again treated with 0.1 % (wt/vol) mercuric chloride solution for 5 minutes, rinsed 3-4 times with sterile distilled water and transferred to

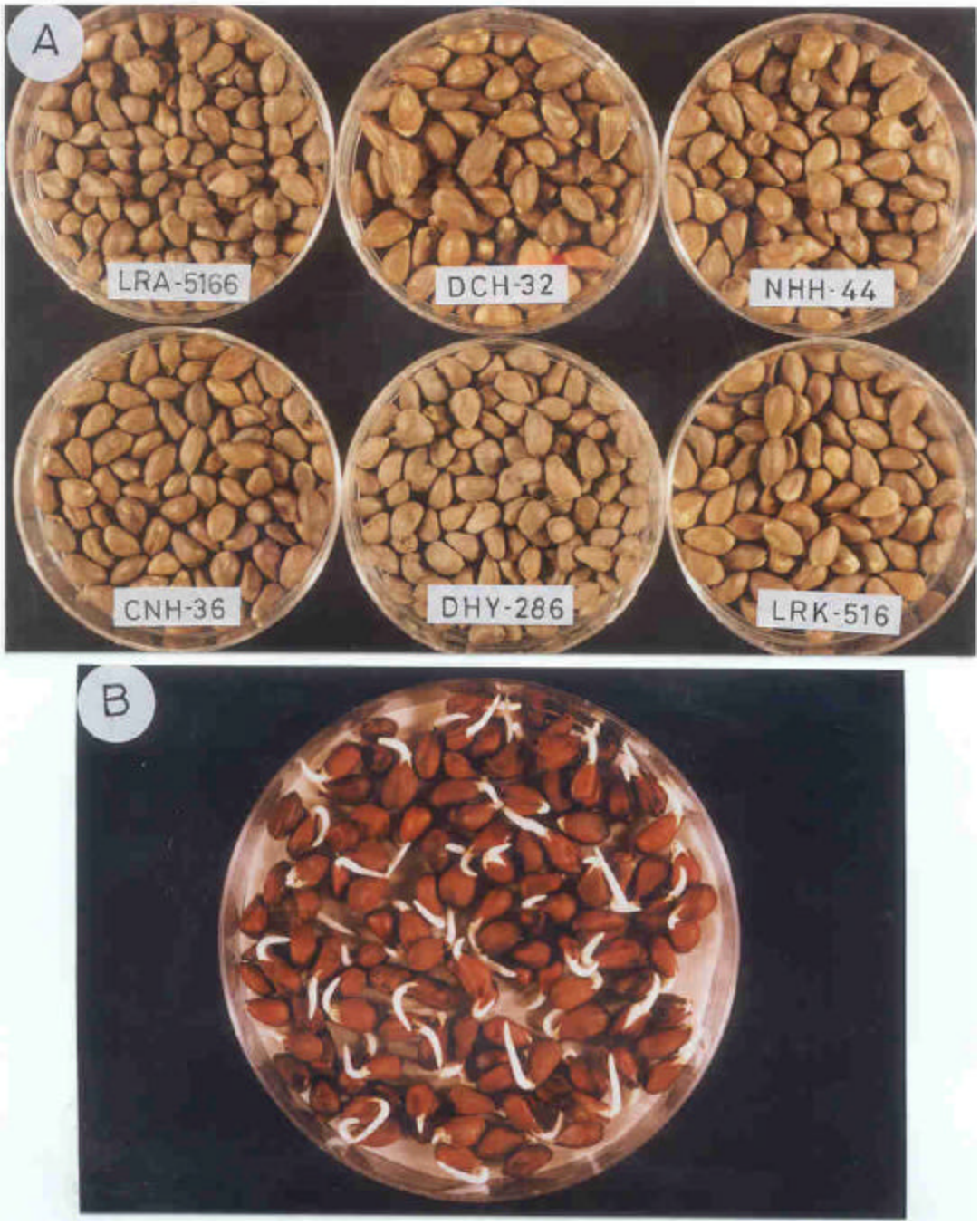
moistened sterile filter paper in petri dishes. Seeds were incubated in the dark for 48 hours for germination (Fig. 2.1B).

Fig. 2.1

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

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

Fig 2



2.6.2. *In vitro* germination of seeds

To obtain high frequency *in vitro* seed germination, conditions like presoaking treatment of sterilized seeds, two basal media and addition of charcoal in the medium were tested. Details of these experiments have been given in chapter 3 and chapter 4.

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.

The number of explants used in each experiment and the number of times each experiment was repeated has been mentioned in materials 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 or three-way analysis of variance (Snedecor & Cochran 1967; Steel and Torrie 1987).

2.9. Culture conditions

The cultures were incubated in a culture room at 25 ± 2° C in dark or light (40 µ mol $m^{-2}s^{-1}$) and in a Growth chamber (HERAEUS VÖTSCH, Germany) at a light intensity of (40 µ mol $m^{-2}s^{-1}$). The details of the incubation conditions have been mentioned in respective sections separately.

2.10. Hardening of plantlets

The shoots derived from various explants were rooted *in vitro*. These plantlets were carefully taken out of the test tubes and gently washed under tap water to remove the gelling agent and the medium sticking to the roots. The plantlets were dipped in 1 % aqueous solution of Bavistin®, a systemic fungicide (BASF, India) for 10-15 min and then washed with tap water. The treated plantlets were transferred to 8 cm earthen pots containing a mixture of autoclaved soil: sand (1:1). The pots were covered with polypropelene bags to prevent the rapid loss of humidity and were kept in greenhouse. 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 particle bombardment mediated transformations and maintenance of the transformed callus line has been described in chapter 6.

Chapter 3

In vitro propagation and plant regeneration from embryo axis explants

3.1. Introduction

Cotton is generally propagated by seeds. The varieties suffer rapid deterioration due to natural crossing and through mechanical mixtures during ginning. (Bajaj 1998). Propagation of cotton plants by vegetative method can be achieved but it is laborious, time consuming and the rate of multiplication is very slow (Parmar *et al.* 1978).

Plant regeneration through organogenesis or somatic embryogenesis is a critical step for the success of any crop improvement program through biotechnological means. Also a simple, rapid and efficient plant regeneration protocol is a major pre-requisite for crop improvement by genetic transformation methods. Low regeneration frequency, chimeras resulting from a group of cells, somaclonal variations and chromosomal abnormalities are some of the limitations of *in vitro* regeneration of plants particularly through somatic embryogenesis via callus phase. Also optimization of a reliable protocol with high frequency regeneration is time consuming and demands a great deal of experimentation (Brar *et al*. 1994; McKently *et al*. 1995). To overcome these limitations, regeneration of plants *via* pre-existing meristems has been used as an alternative approach (Agrawal *et al*., 1997; Saeed *et al*. 1997). The method also results in the development of true to type plants independent of genotypes. *In vitro* propagation can be achieved by inducing the pre-existing meristems to grow and multiply.

3.1.1. *In vitro* plant regeneration from embryo axis explants

At the time of initiation of this study, only two reports of *in vitro* plant regeneration of cotton using embryo axis explants derived from germinated seeds were available. Gould *et al*, 1991 developed single shoots from embryo axis explants, while McCabe and Martinell (1993) described plant regeneration from the explants in the protocol of cotton transformation by particle bombardment method. After that, several reports (Morre *et al*. 1998; Zapata *et al*. 1999) have been published including one from our laboratory (Agrawal *et al*., 1998). Except Morre *et al*. (1998), in all other reports, embryo axis explants developed only single shoot per explant. Zapata *et al* (1999) could obtain plant regeneration from the shoot apices of 3-4 days germinated seeds of 10 cotton cultivars. From our laboratory, the positive effect of Cefotaxime (an antibiotic) on promotion of shoot growth and plant regeneration from embryo axis explant has been reported. (Agrawal *et al.* 1998)

The plant regeneration protocol from embryo axis explant is advantageous since it is cultivar independent and can be utilized in genetic transformation studies with a low

risk of tissue induced genetic variations. Therefore, the main aim of the present study was to develop a plant regeneration system using embryo axis explants of six Indian cultivars of cotton. The present chapter describes:

1. a simple method to regenerate plants from intact and longitudinally split embryo axis explants.
2. a clonal propagation method from shoot tip and axenic nodal segment explants of *in vitro* shoots obtained from intact and longitudinally split embryo axis explants.
3. a protocol for induction and proliferation of multiple shoots and regeneration of plants from plumular axis explants.

3.2. Materials and Methods

3.2.1. Plant material

Delinted seeds of six Indian cultivars of cotton viz. CNH-36, DCH-32, DHY-286, LRK-516, LRA-5166 and NHH-44 were surface sterilized as described in chapter 2.

3.2.2. Germination of seeds

3.2.2.1. Effect of pre-soaking treatment of seeds on germination

Experiments were carried out with all the above mentioned six cultivars. After surface sterilization (as described in chapter 2), seeds were soaked in sterile distilled water or in sterile suspension of activated charcoal [0.25% charcoal (w/v) in distilled water] for 2 or 4 h. After that, the water was drained and seeds were washed 2-3 times with sterile distilled water. These were then transferred to moistened filter papers in pre-sterilized petri dishes (85 mm). The petri dishes were sealed with cling wrap film. The number of seeds with radicle emergence were scored after 48 h of incubation in dark at 25 ± 2 °C.

3.2.3. Embryo axis derived explants

Decotyledonated embryo axis explant (Fig 3.1A, a) was isolated with a mild squeeze of germinated seed and was used as an intact embryo axis explant. In another set of experiments, this intact explant was longitudinally split and the two halves were used as explants (Fig 3.1A, b) (termed hereinafter as split embryo axis explants). Yet in another experiment, the radicle part of the intact embryo axis was cut and discarded and the remaining axis (hereinafter referred to as plumular axis) was used for regeneration studies (Fig.3.1 B). These three explants (viz. intact embryo axis, split embryo axis and the plumular axis) were used in the present study.

In a preliminary set of experiments, sprouting response of intact and split embryo axis of six cotton cultivars was evaluated. The explants were cultured on Murashige and Skoog 1962 (MS) salts and vitamins (hereinafter referred to as MS basal medium) with or without 0.25 % activated charcoal and incubated at 25 ± 2 °C or 30 ± 2 °C.

The method of clonal propagation with apical shoot and the nodal segments from in *vitro* grown shoots derived from the above explants is mentioned in the following sections.

3.2.3.1. Sprouting of intact embryo axis explants

Sprouting of intact embryo axis explants of the above mentioned six cultivars was tested. The explants were cultured in test tubes containing 20 ml of MS basal medium

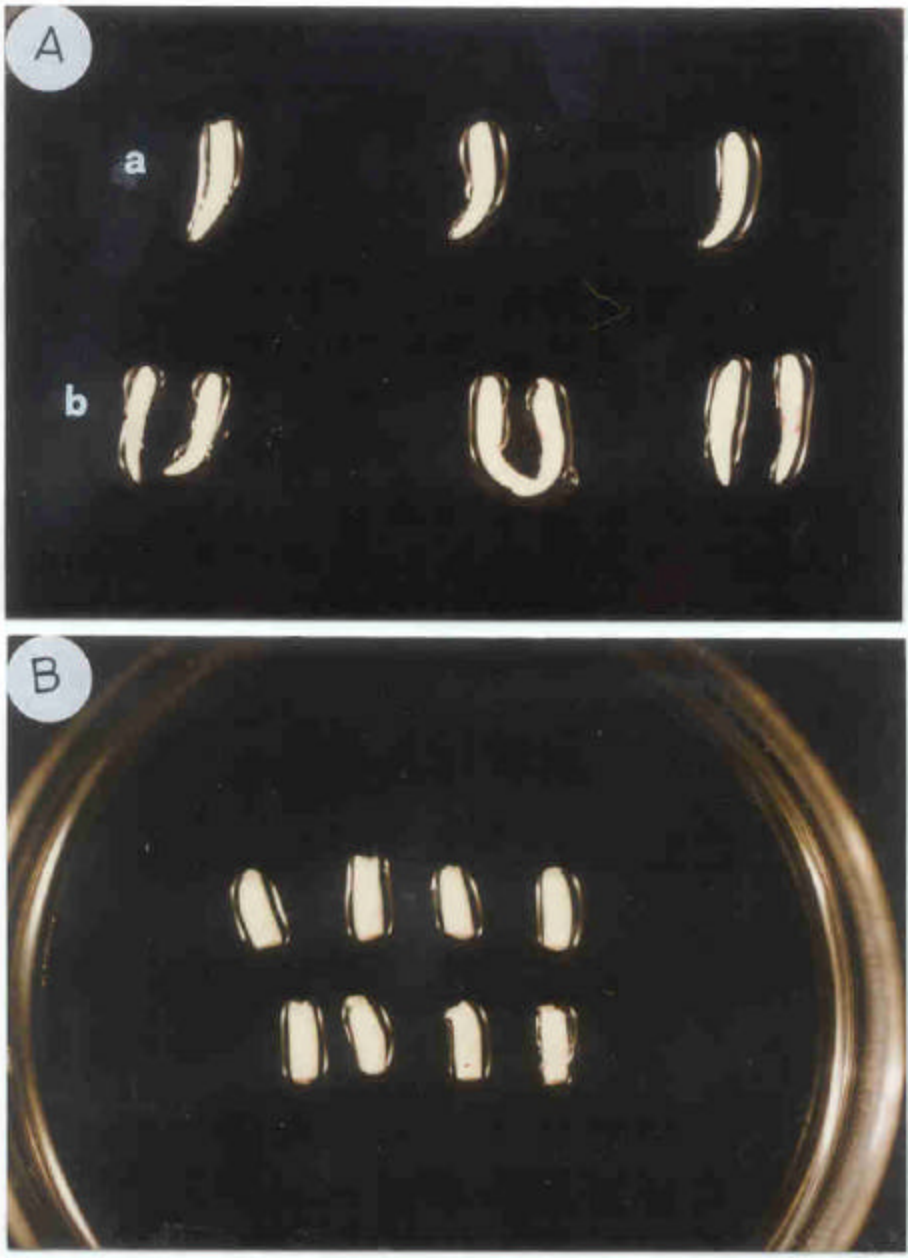
Fig 3.1

A. a. Intact embryo axis explants,

b. Longitudinally split embryo axis explants.

B. Plumular axis explants.

Fig. 3.



supplemented with 2 % sucrose, 0.65 % agar with or without 0.25 % activated charcoal. The cultures were incubated at two different temperatures i.e 25 ± 2 ^{o}C or 30 ± 2 ^{o}C under a light intensity 40 µ mol $m^{-2}s^{-1}$. The number of intact embryo axes that differentiated into plantlets were scored after four weeks of incubation. The experiment was conducted twice using 40 explants of each cultivar. Subsequent experiments were carried out using the same batch of seeds.

3.2.3.2. Effect of activated charcoal and incubation temperature on plantlet formation from longitudinally split embryo axis

In this experiment, embryo axes of the above mentioned six cultivars were split longitudinally into two equal halves without microscope visually. Each half was considered as an independent explant. These were cultured in pairs. A pair of explants were placed horizontally in each culture tube containing 20 ml of MS basal medium with or without 0.25% activated charcoal. As in our previous experiment with intact embryo axis, split axes were also incubated at 25 ± 2 ^{o}C or 30 ± 2 ^{o}C under a 16 h photoperiod at a light intensity of 40 µ mol $m^{-2}s^{-1}$. Observations like bud break, shoot or shoot and root formation and shoot length were recorded after 4 weeks of incubation.

3.2.3.3. *In vitro* propagation of apical shoot and nodal segments

The apical shoots (1.5 to 2 cm in length) excised from *in vitro* shoots developed after 4 weeks of incubation in the above experiments with *in vitro* intact and longitudinally split embryo axis explants were placed vertically in 250 ml flasks (2 apical shoots per flask) containing 50 ml of MS basal medium supplemented with 2 % sucrose and 0.65 % agar. The cultures were incubated at 30 ± 2 ^{o}C under 16 h photoperiod at a light intensity of 40 µ mol $m^{-2}s^{-1}$. After four weeks of incubation, the elongated apical shoots consisting of 3-4 nodes were aseptically removed from the medium and from each such shoot, 2 – 3 nodal segments were excised. These nodal segments were cultured on MS basal medium supplemented with or without BA 0.1 mg/l or kinetin 0.1 mg/l. Shoots developed from axils of the nodal segments were excised and further cultured in 250 ml flasks containing fresh MS basal medium and incubated as described above. Bud break in the nodal segments was recorded after four weeks of incubation. The experiment was repeated twice with twenty explants for each hormonal treatment.

3.2.3.4. Induction of multiple shoots from plumular axis explants

In an initial set of experiments, the effect of two basal media (MS salts and vitamins; MS salts and B_5 vitamins) on sprouting of plumular axis explants (embryo axis

devoid of radicle) was tested. The optimum basal medium (MS salts and B_5 vitamins) was then supplemented with different phytohormones at various concentrations to assess the morphogenetic response in plumular axis explants of cultivar LRK-5166. The identified optimum phytohormones thereafter were evaluated for induction of multiple shoots from plumular axes of three cultivars LRK-5166, DCH-32 and DHY-286. All the media were supplemented with 2 % sucrose and 0.65 % agar (Hi-media, India) and the pH was adjusted to 5.8 before autoclaving. Cultures were incubated at 30 ^{o}C under cool white fluorescent lights at 40 μE m^{-2} S^{-1} with a 16 h photoperiod.

3.2.3.4.1. Effect of basal medium on sprouting of shoots from plumular axis explants

To test the sprouting of plumular axis explants, 100 explants of each cultivar DCH-32, DHY-286 and LRA-5166 were cultured in petri dishes containing semi-solid MS basal medium or MS salts and B_5 vitamins. The number of explants forming shoots was scored after 4 weeks of incubation.

3.2.3.4.2. Effect of various phytohormones on morphogenetic response of plumular axis explants

The influence of various phytohormones on morphogenetic response of plumular axis explants was tested. The plumular axes of the cultivar LRA-5166 were cultured in test tubes containing MS salts + B_5 vitamins and supplemented with TDZ (0.0 - 20.0 mg/l) or 2, 4, 5 - T (0.0 - 20.0 mg/l) or 2, 4 - D (0.0 - 20.0 mg/l) or BA (0.0 – 20 mg/l) or a combination of BA (0.1-2.0 mg/l) and NAA (0.01-0.05 mg/l). The cultures were sub-cultured on fresh medium after 3 weeks. The response of explants was recorded after 6 weeks of incubation.

3.2.3.4.3. Effect of BA and NAA on induction of multiple shoots from plumular axis explants

In this experiment, three cultivars DCH-32, LRK-5166 and DHY-286 were tested for induction and proliferation of multiple shoots. The plumular axis explants were inoculated in petri dishes (85 mm) containing 30 ml of medium constituting of MS salts and B_5 vitamins supplemented with or without BA (0.05-3.0 mg/l) alone or in combination with NAA (0.01-0.03 mg/l) (optimized in the above experiment). The cultures were incubated under 16 h photoperiod at 30 ^{o}C at a light intensity of 40 μ mol $m^{-2}s^{-1}$ for 3 weeks. The elongated apical shoots and developed roots were excised and discarded. These decapitated explants were re-cultured on fresh medium for further 3 weeks. Thereafter, the cultures were transferred to 100 ml Erlenmeyer flasks containing

30 ml of fresh medium. The number of shoots developed per explant was scored after 6 weeks of incubation. The shoot clusters were then transferred to 250 ml Erlenmeyer flasks containing 100 ml of fresh medium. The number of shoots developed per explant was recorded after 10 weeks of incubation. The experiment was repeated thrice with 10 replicates for each treatment. Data was statistically analyzed by two-way analysis of variance (ANOVA). The number of explants responded and the number of shoots developed per responding explant was the parameter used to determine the optimum medium for the induction and proliferation of multiple shoots from plumular axis explants.

3.2.4. *In vitro* rooting of shoots

The shoots from intact embryo axes and the axenic nodal segments rooted simultaneously in the same medium (MS basal medium with or without 0.25 % activated charcoal and 2% sucrose) after four weeks.

Elongated shoots (3 - 4 cm) from the multiple shoot bunches (obtained from plumular axis explants) were excised (20 shoots of each cultivar) and transferred to MS basal medium devoid of phytohormones for rooting.

3.2.5. Hardening of plantlets and transfer of plants to soil.

Rooted shoots obtained from the split embryo axes (20 plantlets from each cultivar) and axenic nodal segments (10 plantlets from each cultivar) and multiple shoots (50 plantlets) were transferred to earthen pots (8 cm) containing a mixture of sand, soil (1:1) and kept under greenhouse conditions for hardening. Rooted shoots were hardened as described in chapter 2, section 2.10. After 4 weeks, hardened plants were transferred to field.

3.3. Results and discussion

The data collected on seed germination of six Indian cultivars of cotton, the influence of activated charcoal and the incubation temperature on the frequency of plant development from intact and split embryo axes have been described in this chapter. A method of clonal propagation using axenic nodal segments derived from *in vitro* shoots derived from intact and split embryo axes of six cotton cultivars has been described. Also induction of multiple shoots from plumular axis explants of three cultivars of cotton has been described in this chapter.

3.3.1. Effect of pre-soaking treatment on seed germination

Results on the effect of pre-soaking treatment of seeds with water and charcoal suspension of six cultivars of cotton on germination is presented in Table 3.1. In all the six cultivars, the maximum seed germination was obtained on pre-soaking in charcoal suspension for 4 h. Out of the two time periods tested, the maximum seed germination on pre-soaking in distilled water was recorded when seeds were pre-soaked for 4 h (49.3 – 62.7 %) compared to 2 h (43.3 – 52.3 %) (Table 3.1). A wide variation in germination percentage was observed between the cultivars under all the four conditions tested. Activated charcoal is a known adsorbent used in tissue cultures to remove growth retarding phenolics released during incubation period (Fridborg et al., 1978), however, its positive influence on seed germination in cotton has not been reported earlier.

There are several reports available where germination of cotton seed has been reported by different methods. In one approach, seed coat was removed before seed was cultured in MS basal medium at 28 ^{o}C in light (Zapata *et al*., 1999, Voo *et al*., 1991, Kumar *et al*., 1998), while in other approach, surface sterilized seeds were directly placed on MS salts and B5 Vitamins for germination (Zhang *et al*., 2000, Finer 1988). Yet in another report, Trolinder & Goodin (1988); Davidonis & Hamilton (1983) obtained cotton seed germination on moistened sterile filter papers in petri dishes. Hemphill *et al*., (1998) obtained cotton seed germination by pre-soaking them in sterile water for 3 h as reported here by us. As evident from the results, charcoal suspension and presoaking treatment for 4 h drastically improved the germination % of cotton seeds.

Tables 3.1: Effect of pre-soaking treatment of seeds on germination in six cotton cultivars.

Cultivars	Germination %			
	Distilled water		Charcoal suspension*	
	2h	4h	2h	4h
CNH-36	43.7 ± 10.0	59.0 ± 7.9	61.0 ± 6.9	64.7 ± 9.2
DCH-32	52.3 ± 7.4	62.7 ± 6.8	69.3 ± 3.1	82.3 ± 4.9
DHY-286	49.0 ± 7.8	54.3 ± 8.1	62.7± 6.1	72.7 ± 6.8
LRA-5166	43.3 ± 5.0	53.3 ± 7.4	58.3 ± 6.7	70.0 ± 5.0
LRK-516	46.7 ± 3.2	49.3 ± 5.1	61.7 ± 4.0	73.7 ± 5.1
NHH-44	51.3 ± 7.2	57.7 ± 2.5	59.7 ± 8.4	77.0 ± 7.5

*Charcoal suspension = 0.25% charcoal in distilled water

3.3.2. Plant regeneration from intact and split embryo axis explants

This section describes sprouting of shoots, influence of charcoal and incubation temperature on plant regeneration from intact embryo axis and split embryo axis explants. Also, the clonal propagation by using shoot tip and axenic nodal segments has been described.

3.3.2.1. Sprouting of intact embryo axis explants

This section describes results on sprouting and shoot formation from the intact embryo axis explants (6 - 8 mm long) cultured under two temperatures i.e. 25 ± 2 °C or 30 ± 2 °C in MS basal medium with or without charcoal. The maximum sprouting was observed in the basal medium with charcoal at both the temperatures irrespective of the cultivars (Table 3.2). The frequency of sprouting was higher at 30 ± 2 °C (67.5 - 83.3 %) compared to at 25 ± 2 °C (48.8 - 81.3 %). Among the six cultivars, the maximum response was observed in cultivar NHH-44 (83.3 %) with charcoal when incubated at 30 ± 2 °C. Among the two media tested, there was only a marginal difference in sprouting response at 30 ± 2 °C (Table 3.2). However, the shoots in the basal medium containing charcoal were found to be healthier with thicker stems and bigger leaves (Fig. 3.2 A). This positive influence of charcoal may be due to adsorption of phenolics or other compounds leached into the medium by sprouting cultures (Madhusudhanan and Rahiman, 2000).

Table 3.2: Effect of activated charcoal and incubation temperature on sprouting in the intact embryo axis explants.

Cultivars	Incubation at 25 °C		Incubation at 30 °C	
	MS	MS + Char	MS	MS + Char
CNH-36	48.8 ± 5.3	60.0 ± 10.6	67.5 ± 3.5	70.0 ± 3.5
DCH-32	60.0 ± 3.5	68.8 ± 1.8	68.8 ± 5.3	76.3 ± 1.8
DHY-286	62.5 ± 10.6	72.5 ± 3.5	72.5 ± 3.5	80.0 ± 3.5
LRA-5166	65.0 ± 7.1	68.8 ± 8.8	77.5 ± 7.1	78.8 ± 1.8
LRK-516	61.3 ± 1.8	75.0 ± 3.5	81.3 ± 8.8	81.3 ± 5.3
NHH-44	67.5 ± 7.1	81.3 ± 5.3	83.3 ± 5.3	82.5 ± 3.5

3.3.2.2. Effect of activated charcoal and incubation temperature on plantlet formation from split embryo axis explants

The results of this experiment are presented in Table 3.3. Two types of responses were observed when split embryo axis explants were cultured on MS basal medium with or without charcoal at two different temperatures. (1) Explants formed both shoots and roots which indicates that both the root and shoot meristems were present in these explants either partially or fully and were regenerative. (2) Explants formed only either shoot or root indicates that the incision was oblique. Eventually only those explants which formed shoots (with or without roots) were used for establishment of shoot cultures or plants.

Incorporation of charcoal in MS basal medium and incubation of explants at 25 ± 2 °C induced formation of roots and shoots simultaneously at higher percentages than MS basal medium alone in all cultivars except DCH-32. However, the overall frequency of shoot formation from explants in the basal medium with charcoal was lower than without charcoal due to a decrease in the number of explants forming only shoots. The decrease in the number of explants forming shoots alone in MS basal medium without charcoal at 30 ± 2 °C (Table 3.3) compared to 25 ± 2 °C (Table 3.3) is quite significant indicating that more number of explants produced both shoots and roots (Table. 3.3). The total number of explants forming shoots were significantly higher at 30 ± 2 °C compared to 25 ± 2 °C in MS medium with or without charcoal in all the cultivars (except DCH-32 and NHH 44 in MS medium alone). Significant increase in the frequency of shoot formation has been

observed in MS basal medium with charcoal compared to the medium without charcoal at 30 ± 2 °C in all the cultivars, except LRA 5166 and LRK 516. The frequency of shoot formation in explants cultured in media with or without charcoal and the influence of two different incubation temperatures has been calculated from the total number of responding explants forming shoots with or without roots (Table 3.3). The statistical analysis, three-way analysis of variance of data as described by Steel and Torrie (1987) has been presented in (ANOVA) Table 3.3.

Shoot length was measured with a scale to assess the vigor of the plantlets. In general the positive influence of charcoal in the medium and incubation temperature of 30 ± 2 °C on shoot length was observed (Fig 3.2 B & Fig. 3.3). Similarly, Morre *et al* (1998) also reported better growth of cotton cultures at 30 ± 2 ^{o}C. With the exception of DHY-286, incorporation of 0.25% charcoal in the medium enhanced the morphogenetic response of all other cultivars. The beneficial effects of charcoal in plant tissue cultures have been reported earlier (Fridbourg *et al*.1978, Wann *et al*. 1997, Madhusudhanan and Rahiman 2000). To the best of our knowledge, this synergistic effect of charcoal with increase in incubation temperature on morphogenesis has not been reported earlier.

Fig. 3.2

A. Shoot regeneration in intact embryo axis explants on MS basal medium and MS basal medium containing charcoal incubated at 30 ^{o}C.

B. Shoot regeneration from the split embryo axis explants of six cultivars in medium containing charcoal and incubation on at 30 °C.

Fig. 3.2

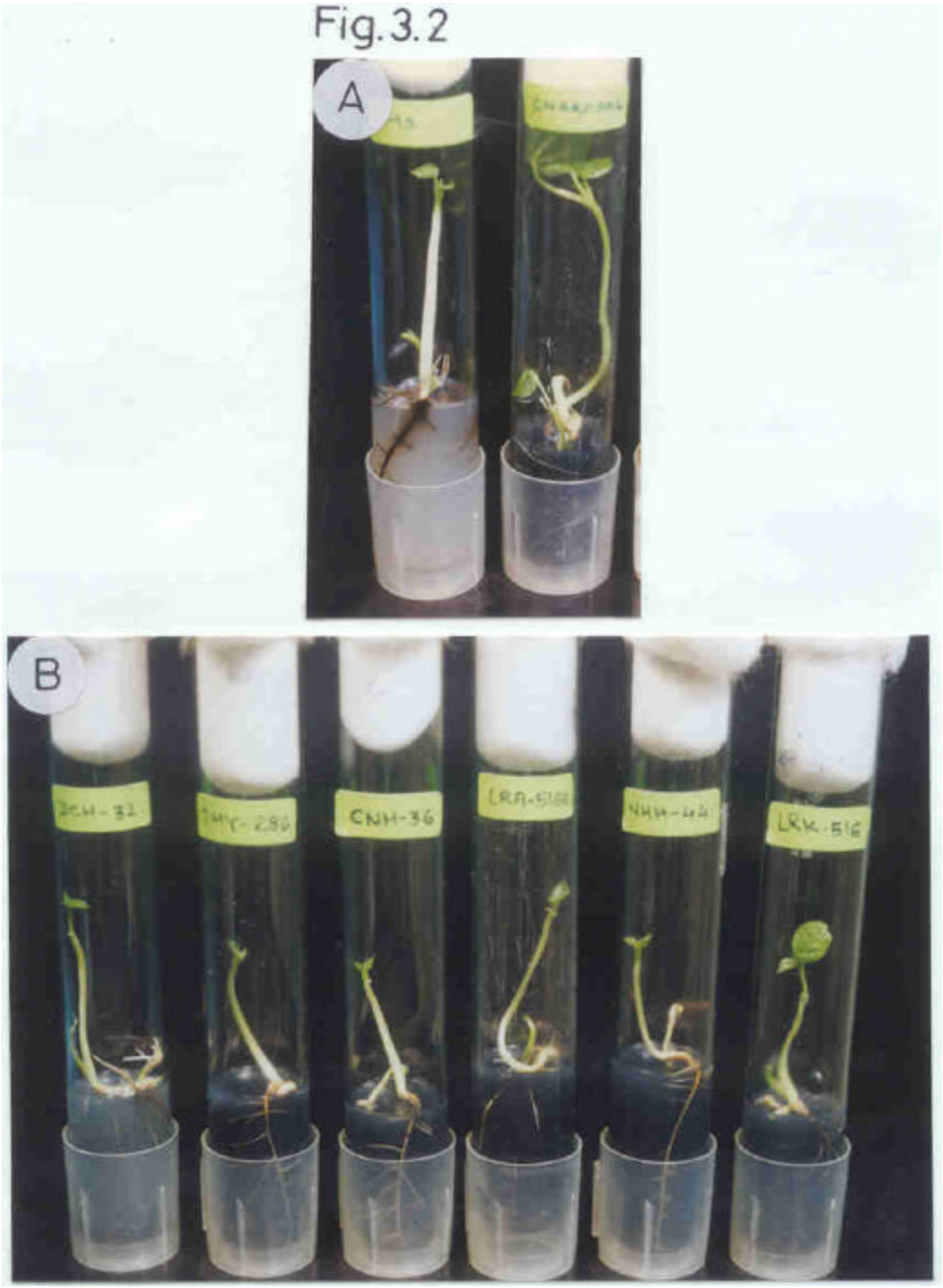


Fig. 3.3

A. Effect of charcoal & incubation temperature (25 ^{o}C) on shoot length in split embryo axis explants.

B. Effect of charcoal and incubation temperature (30 ^{o}C) on shoot length in split embryo axis explants.

Fig. 3.3

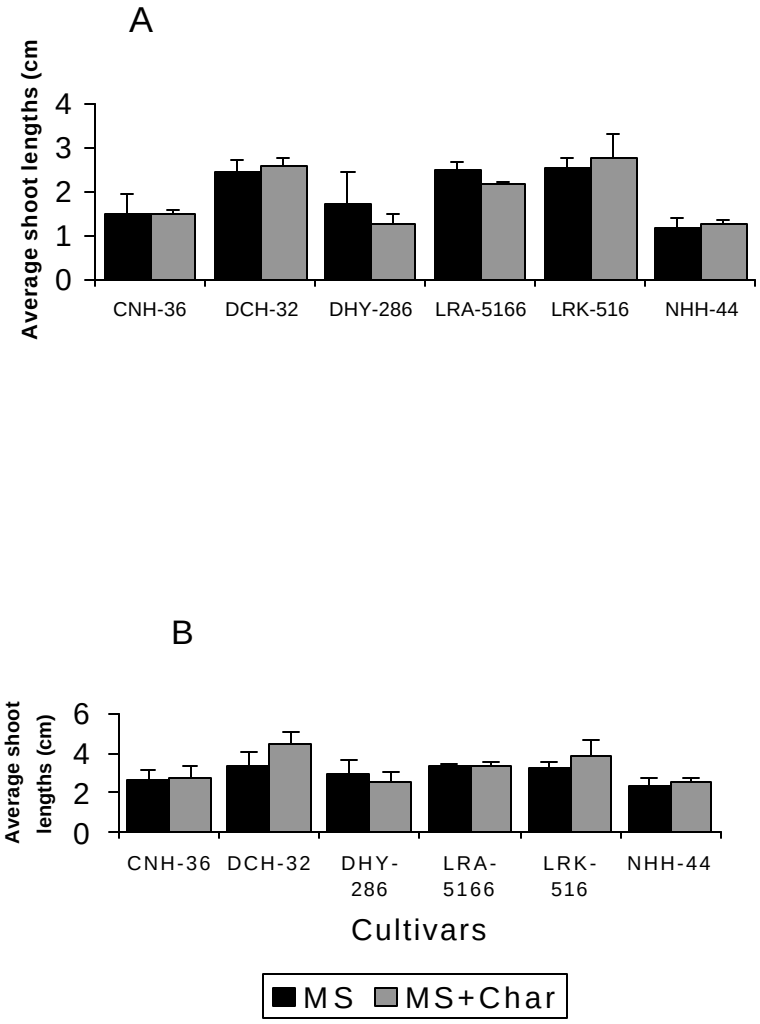

A
Average shoot lengths (cm
4
3
2
1
0
CNH-36
DCH-32
DHY-286
LRA-5166
LRK-516
NHH-44
B
Average shoot lengths (cm)
6
4
2
0
CNH-36
DCH-32
DHY-286
LRA-5166
LRK-516
NHH-44
Cultivars
MS
MS+Char

Table 3.3: Effect of activated charcoal and incubation temperature on the frequency of shoot/root formation in split embryo axis explants of six cotton cultivars.

Cultivars	Media	Incubation at 25 °C			Incubation at 30 °C		
		No. of explants	Explants forming shoots (%)	Explants forming shoots & roots (%)	No. of explants	Explants forming shoots (%)	Explants forming shoots & roots (%)
CNH-36	MS	119	17	37	66	6	45
	MS+Char	42	0	57	95	4	66
DCH-32	MS	60	16	53	60	0	54
	MS+Char	62	0	37	76	0	68
DHY-286	MS	120	14	32	67	9	40
	MS+Char	40	0	44	112	3	53
LRA-5166	MS	59	27	27	60	4	50
	MS+Char	58	0	31	108	0	53
LRK-516	MS	130	22	35	64	9	53
	MS+Char	124	3	37	138	0	54
NHH-44	MS	119	17	56	60	5	48
	MS+Char	40	0	62	115	6	56

Data scored after four weeks of incubation.
MS = Murashigae and Skoog's salts and vitamins.

Char = 0.25 % Activated charcoal

ANOVA Table for Table 3.3.

Source	Df	MSS	F-Value	
Block	2	1191.32	3.72	*
Treatment	23	498.32	1.56	ns
G	5	223.11	0.70	ns
T	1	1803.25	5.63	*
M	1	1190.89	3.72	ns
G*T	5	53.59	0.17	ns
G*M	5	19600.40	61.20	*
T*M	1	5265.92	16.44	*
G*T*M	5	900.23	2.81	*
Error	46	320.28		
Total	71			

*Significant at $p \leq 0.05$
C- cultivar, T- temperature, M- medium

3.3.2.3. *In vitro* propagation of apical shoots and nodal segments

This section describes the results obtained in two independent experiments: One with apical shoots and another with nodal segments derived from *in vitro* grown shoots. Apical shoots were cultured on MS basal medium alone, while nodal segments were cultured on MS basal medium supplemented with or without BA or Kinetin. Responses in terms of bud break in nodal segments and growth in apical shoots were recorded.

The apical shoots cultured vertically in flasks elongated, developed 3 - 4 nodes and induced roots (Fig 3.4 A). From each of these elongated shoots, further 3 - 4 nodal segments and one apical shoot could be excised and sub-cultured on fresh medium. Thus a cycle of nodal segments and apical shoots could be maintained up to 10 subcultures from one apical shoot.

In all the six cultivars, nodal segments developed shoots in all the media combinations tested (Table 3.4). Irrespective of the cultivar, the frequency of response was higher (68 to 82 %) on the medium devoid of phytohormones compared to media supplemented with BA or Kin. In controls, the maximum number of nodal segments developed shoots in cultivar LRA-5166 (82.2 %) followed by NHH-44 (81.2%), DHY-286 (81%), LRK-516 (74.4 %), DCH-32 (73.6%) and CNH-36 (67.7%) (Table 3.4). Single shoot and roots developed in each nodal segment on four weeks of incubation (Fig 3.4 B). BA or Kinetin at lower concentrations (0.1 mg/L) decreased the bud break response drastically. Similar to the present study, inhibitory effect of lower concentrations of Kinetin (Gould *et al*. 1991) or BA (Zapata *et al* 1999) in formation of shoots in the apical shoot meristems of cotton has earlier been reported. Between the two cytokinins, BA supported formation of 2 shoots in cultivars DCH-32, DHY-286 and NHH-44. However, these shoots remained stunted. Elongation of these stunted shoots could be achieved on transferring them to the basal medium devoid of phytohormones. Similar to our study, clonal propagation by nodal cuttings and apical shoots excised from in vitro grown shoots in blueberry (Cohen and Elliott 1979) and *Leucaena* (Dhawan and Bhojwani 1985) have been reported.

Fig. 3.4

A. Elongation and rooting in the apical shoots cultured vertically in flask containing MS basal medium.

B. a. Bud break in nodal segment,

b. & c. Elongation of axillary shoots,

d. rooting of the axillary shoot.

Fig 3 4

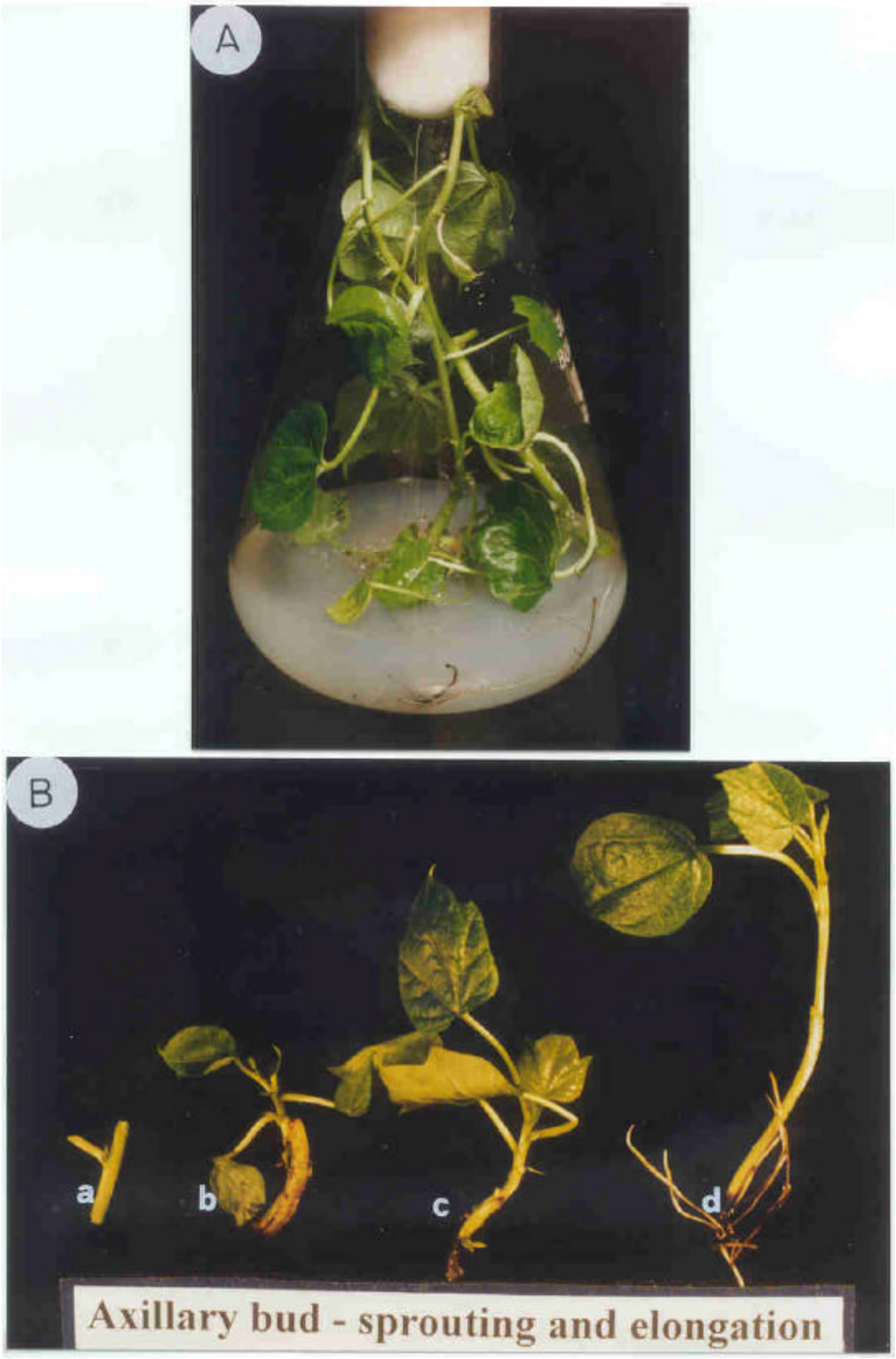


Table.3.4: Shoot development in nodal segments from *in vitro* grown shoots.

Cultivar	BA (mg/l)	Kinetin (mg/l)	Explant response (%)	Nature of response
CNH-36	0.0	0.0	67.7 ± 6.4	Single shoot
	0.1	0.0	25.5 ± 6.3	Single stunted shoot
	0.0	0.1	22.5 ± 3.5	Single stunted shoot
DCH-32	0.0	0.0	73.6 ± 2.0	Single elongated shoot
	0.1	0.0	33.3 ± 2.4	1-2 stunted shoots
	0.0	0.1	29.0 ± 3.7	Single stunted shoot
DHY-286	0.0	0.0	81.0 ± 4.5	Single elongated shoot
	0.1	0.0	28.3 ± 4.7	1-2 stunted shoots
	0.0	0.1	23.0 ± 2.8	Single stunted shoot
LRA-5166	0.0	0.0	82.2 ± 6.3	Single shoot
	0.1	0.0	31.3 ± 8.8	Single stunted shoot
	0.0	0.1	25.0 ± 3.9	Single stunted shoot
LRK-5166	0.0	0.0	74.4 ± 8.0	Single shoot
	0.1	0.0	27.8 ± 7.9	Single stunted shoot
	0.0	0.1	27.8 ± 7.9	Single stunted shoot
NHH-44	0.0	0.0	81.7 ± 2.4	Single elongated shoot
	0.1	0.0	35.0± 7.1	1-2 stunted shoots
	0.0	0.1	33.4 ± 4.8	Single stunted shoot

3.3.3. *In vitro* induction of multiple shoots from plumular axis explants

The following section describes the results obtained on the plumular axis explants sprouting response in the basal medium, the effect of different phytohormones and the effect of BA and NAA on induction of multiple shoots from plumular axis explants.

3.3.3.1. Effect of basal medium on sprouting of shoots from plumular axis explants

This study was carried out to assess the effect of two basal media [(MS salts + vitamins hereinafter referred as MS basal medium) and MS salts + B5 vitamins] on sprouting of shoots from plumular axis explants. This experiment was done with cultivars LRA-5166, NHH - 44 and DCH - 32 as these are most popular cultivars grown in different southwestern states of India. In all the three cultivars tested, MS salts + B5 vitamins (Table 3.5) supported the maximum percentage of sprouting. Therefore, in all our further experiments with plumular axes, MS salts + B_5 vitamins was used as a basal medium. The use of MS salts and modifications in the constituents of vitamins in basal media for *in vitro* plant regeneration from pre-existing meristems has been reported for cotton (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 shoot regeneration (58%) from shoot apices of cotton by culturing the explants on MS salts + modified B_5 vitamins without phytohormones.

Table.3.5: Effect of basal media on sprouting of shoot from plumular axis explants.

Medium	Number of explants sprouted (%)		
	DCH-32	DHY-286	LRA-5166
MS salts & vitamins	83.5 ± 6.4	81.0 ± 7.1	77.0 ± 4.2
MS salts & B5 vitamins	90.0 ± 7.1	93.0 ± 4.2	81.5 ± 5.0

3.3.3.2. Effect of various phytohormones on morphogenetic response of plumular axis explants

This experiment was carried out initially with the objective of induction of embryogenesis in plumular axis explants, with one cotton cultivar (LRA-5166). The plumular axis explants cultured in medium containing MS salts + B_5 vitamins and supplemented with a wide range of phytohormones, 2, 4 - D, 2,4,5 - T, TDZ, BA and combinations of BA + NAA, showed varied morphogenetic responses (Table 3.6).

Though somatic embryogenesis could not be induced in the present set of experiments, morphogenetic responses observed with different phytohormones have been described in Table 3.6.

Plumular axis explants cultured in the basal medium containing 2, 4 – D (0.001 – 0.1 mg/l) formed single shoots and developed roots. Explants turned brown on higher concentrations of 2,4-D (more than 0.1 mg/l), while at lower 2,4-D concentrations, formation of white, loose callus was observed. Explants cultured in the basal medium containing 2, 4, 5 – T (0.001 – 0.1 mg/l) had similar morphogenetic response as in 2,4-D. Lower concentrations of TDZ (0.001 - 0.05 mg/l) supported formation of higher number of shoots (2-3 shoots per explant). However, the explants swelled and induced callus. TDZ at higher concentrations (1.0 – 5.0 mg/l) did not support shoot formation but induced only callus. At still higher TDZ concentrations (10 – 20 mg/l), the explants turned brown and did not show any morphogenetic response. Of all the phytohormones tested, BA was the best. BA at lower concentrations (0.001 – 0.1 mg/l) supported formation of 1-2 shoots, while concentrations (0.5 – 5.0 mg/l) formed 2-3 shoots per explant. However, the explants turned brown at higher concentrations of BA (10.0 – 20.0 mg/l). Morre *et al* (1998) has earlier reported the formation of 2-3 shoots / per plumular axis explant in cotton on incorporation of BA (3.0 mg/l). However, Zapata *et al* (1999) found that BA at 1 mg/l suppressed the shoot regeneration in embryo axis explants. In our study, combinations of BA and NAA (0.1 + 0.02, and 0.5 + 0.03, respectively) supported the formation of 2-3 elongated shoots. Concentrations higher than these resulted in the formation of single stunted shoot (Table 3.6).

From the above study it can be concluded, that BA alone or a combination of BA and NAA are the most potential phytohormones for the induction of multiple shoots from the plumular axes of the cotton cultivar LRA-5166. Therefore, further experiments were carried out to optimize the concentrations of these two phytohormones for induction of multiple shoots in three cotton cultivars viz. DCH-32, DHY-286 and LRA-5166.

Table 3.6: Effect of various phytohormones on morphogenesis of plumular axis explants of cotton cultivar LRA-5166.

Phytohormone	Conc. (mg/l)	Explants response (%)	Nature of response
2,4-D	0.001	78.3	Single elongated shoot, well developed roots
	0.005	71.6	Single elongated shoot, well developed roots
	0.01	66.6	Single elongated shoot, well developed roots
	0.05	38.3	Single elongated shoot, roots
	0.1	18.0	Single stunted shoot, roots, callus
	0.5	100	Explants turned brown
	1.0	100	Explants turned brown
	2.0	100	Explants turned brown
	5.0	100	Explants turned brown
	10.0	100	Explants turned brown
	15.0	100	Explants turned brown
	20.0	100	Explants turned brown
2,4,5-T	0.001	65.0	Single elongated shoot, well developed roots
	0.005	61.0	Single elongated shoot, well developed roots
	0.01	53.3	Single elongated shoot, well developed roots
	0.05	35.0	Single elongated shoot, callus, roots
	0.1	28.3	Single stunted shoot, callus, roots
	0.5	25.0	Single stunted shoot, tip dried
	1.0	18.3	Single stunted shoot, tip dried
	2.0	100	Explants turned brown
	5.0	100	Explants turned brown
	10.0	100	Explants turned brown
	15.0	100	Explants turned brown
	20.0	100	Explants turned brown

Phytohormone	Conc. mg/l	Explants response (%)	Nature of response
TDZ	0.001	61.6	2-3 elongated shoots, callusing, swollen
	0.005	55.0	2-3 elongated shoots, callusing, swollen
	0.01	51.6	2-3 elongated shoots, callusing, swollen
	0.05	51.6	2-3 elongated shoots, callusing, swollen
	0.1	46.6	Callus at shoot tip
	0.5	40.2	Callus at shoot tip
	1.0	38	Profuse Callus at shoot tip, explant swollen
	2.0	25	Profuse Callus at shoot tip, explant swollen
	5.0	25	Profuse Callus at shoot tip, explant swollen
	10.0	100	Explants turned brown
	15.0	100	Explants turned brown
	20.0	100	Explants turned brown
BA	0.001	78.3	1-2 elongated shoots, roots
	0.005	80.0	1-2 elongated shoots, roots
	0.01	83.3	1-2 elongated shoots, roots
	0.05	83.3	1-2 elongated shoots
	0.1	85.0	1-2 elongated shoots
	0.5	88.3	2-3 elongated shoots
	1.0	93.3	2-3 elongated shoots
	2.0	81.6	2-3 stunted shoots
	5.0	46.6	1-2 stunted shoots
	10.0	100	Explants turned brown
	15.0	100	Explants turned brown
	20.0	100	Explants turned brown
BA + NAA	0.5 + 0.01	80.0	1-2 elongated shoots
	0.1 + 0.02	85.0	2-3 elongated shoots
	0.5 + 0.03	50.0	2-3 elongated shoots
	1.0 + 0.05	32.0	Single stunted shoots
	2.0 + 0.05	15.0	Single stunted shoots

3.3.3.3. Effect of BA and NAA on induction of multiple shoots from plumular axis explants

This section describes the induction of multiple shoots from plumular axis explants of three cultivars of cotton. With different concentrations of BAP and BAP+NAA tested, plumular axes induced shoots varying from 1 to 6.04 per explant depending upon the cultivar and the hormone concentration in the basal medium (Table 3.8). Though the number of multiples formed per explant varied among the three cultivars, the maximum number of shoots in all three cultivars was observed on the basal medium supplemented with BAP (0.1 mg/l) + NAA (0.02 mg/l) (Table 3.8). On this medium, 81.66, 80 and 73.33 percent of explants developed multiple shoots in DCH-32, DHY-286 and LRA-5166 respectively (Table 3.7). As for as the number of shoots per explant is concerned, the highest number was recorded in cultivar DCH-32 (6.04 ± 1.38) followed by DHY-286 (4.55 ± 1.51) and LRK-5166 (4.55 ± 1.25). On transfer of these shoot clusters to fresh medium and on incubation for 10 weeks, more than two-fold increase in the number of multiple shoots was observed (Fig. 3.5). The highest number of shoots per explant was recorded in cultivar DCH-32 (15.04 ± 3.07) followed by DHY-286 (11.12 ± 2.80) and LRA-5166 (8.04 ± 2.03).

The medium supplemented with BAP (1.0 mg/l)+NAA (0.03 mg/l) did not support the production of multiple shoots in all three cultivars. BA alone at 0.10 mg/l induced 4.63 ± 1.09 shoots/explant in DCH-32 followed by DHY-286 (3.64 shoots /explant), LRA-5166 (3.1 shoots/explant). BA concentrations higher than 0.1mg/l did not induce multiple shoots in all the three cultivars (Table 3.8).

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 basal medium with BA (0.1 mg/l) + NAA (0. 02 mg/l) was essential to enhance the induction of multiple shoots. Inclusion of BA alone (3 mg/l) in the medium did not induce shoot multiples. In a

Fig. 3.5

Induction of multiple shoots in plumular axis explant of cultivar DCH-32 after ten weeks of incubation.

Fig 3.5

similar experiment, Hemphill *et al*. (1998), obtained only stunted shoots from embryo axes on a medium containing BA 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).

Though plant regeneration in cotton has been achieved from 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 plumular axis explant of an Argentine cotton cultivar cv. Guazuncho II. Therefore these results have enormous significance.

Table 3.7: Percentage response of induction of multiple shoots from plumular axis explants of three Indian cultivars of cotton.

Phytohormone		Explants response (%)		
BA	NAA	Cultivars		
Mg/l	Mg/l	DCH-32	LRA-5166	DHY-286
0.00	0.00	85.00	80.0	85.00
0.05	-	78.33	70.0	71.66
0.10	-	75.00	60.0	63.33
0.50	-	46.66	36.7	53.33
1.00	-	36.66	35.0	36.66
3.00	-	31.66	18.3	20.00
0.05	0.01	71.66	63.3	66.66
0.10	0.02	81.66	73.3	80.00
0.50	0.03	45.00	33.3	43.33
1.00	0.03	33.33	23.3	28.33

Data scored after 12 weeks of incubation.
Experiment repeated twice with thirty replicates.
Critical Difference - 12.12

ANOVA Table for Table. 3.7

Source	Df	SS	MSS	F-Calculated	F-Table value
Total	119	60759.98	510.59	0.50	
Treatment	59	59500.80	1008.49	48.06	1.74
Media (M)	11	54805.79	4982.35	237.41	2.51
Genotype (G)	5	2380.67	476.13	22.69	3.28
G × M	55	2314.34	42.08	2.01	1.80
Error	60	1259.18	20.99		

Table 3.8: Effect of phytohormones on induction of multiple shoots from plumular axis explants of three Indian cultivars of cotton.

Phytohormones		No. of shoots / explant		
BA	NAA	Cultivars		
Mg/l	mg/l	DCH-32	LRA-5166	DHY-286
0.00	0.00	1.98 ± 0.62	1.57 ± 0.58	1.40 ± 0.58
0.05	-	3.24 ± 0.85	2.61 ± 0.95	2.31 ±0.86
0.10	-	4.63 ± 1.09	3.15 ± 0.77	3.64 ± 1.24
0.50	-	1.96 ± 0.64	1.65 ± 0.55	1.55 ± 0.52
1.00	-	1.48 ± 0.51	1.18 ± 0.41	1.29 ± 0.43
3.00	-	1.22 ± 0.45	1.17 ± 0.43	1.18 ± 0.47
0.05	0.01	4.65 ± 0.95	3.12 ± 0.93	3.28 ± 1.10
0.10	0.02	6.04 ± 1.38	4.55 ± 1.25	4.77 ± 1.51
0.50	0.03	2.37 ± 1.05	1.55 ± 0.58	1.76 ± 0.75
1.00	0.03	1.62 ± 0.58	1.29 ± 0.49	1.21 ± 0.46

± = Standard error (s.d)

Data scored after 12 weeks of incubation.
Experiment repeated twice with thirty replicates.
Critical Difference - 0.47

ANOVA Table for Table. 3.8

Source	df	SS	MSS	F-Calculated	F-Table value
Total	119	205.52	1.73	0.50	
Treatment	59	203.65	3.45	111.13	1.74
Media (M)	11	178.56	16.23	522.60	2.51
Genotype (G)	5	14.73	2.95	94.83	3.28
G × M	55	10.37	0.19	6.07	1.80
Error	60	1.86	0.031		

3.3.4. *In vitro* rooting of shoots

All the shoots regenerated from intact embryo axes, split embryo axes and the nodal segments rooted simultaneously in the same culture medium (MS basal medium with or with out 0.25 % activated charcoal) during the incubation of 4 weeks.

Elongated shoots from multiple shoot clusters (derived from plumular axis explants) rooted (Fig 3.6) on MS basal medium devoid of phytohormones on incubation for 3 weeks. The percentage rooting was 90 % in cultivars DCH-32 and DHY-286 and 80 % in cultivars LRA-5166.

3.3.5. Hardening of plantlets and transfer to soil

The plants raised from the split embryo axes survived in soil in low frequency of 30-35%. On transfer of 120 tissue culture plants (20 of each cultivar) to earthen pots, it was observed that the plants were thin and crooked (Fig. 3.7 A). This may be due to partial stem development in the split axis. However, these plants became erect and normal on transfer to the greenhouse for three months (Fig. 3.7 B). Thirty-five plants raised by this method survived and formed bolls on maturation in the field (Fig. 3.7 C).

The rooted shoots (10 plantlets of each cultivar) derived from the nodal segment explants survived (60-80 %) in soil. These plants grew to maturity and developed normal bolls after 4 months.

Survival of the rooted shoots derived from multiple shoot clusters developed from plumular axis explants was found to be 92% after hardening in greenhouse (Fig. 3.8 A). All the plants grew to maturity and developed normal bolls after 4 months of transfer to field (Fig. 3.8 B).

(*Schematic representation of regeneration from explants derived from embryo axis has been given in the following page*).

Fig. 3.6 *In vitro* rooted shoot.

Fig 3.6

Fig. 3.7

A. Plants raised from split embryos and transferred to soil are initially thin and crooked.

B. Erect and normal plant (derived from split embryo axis) after 12 weeks in greenhouse conditions.

C. Mature plant in soil.

Fig 3 7

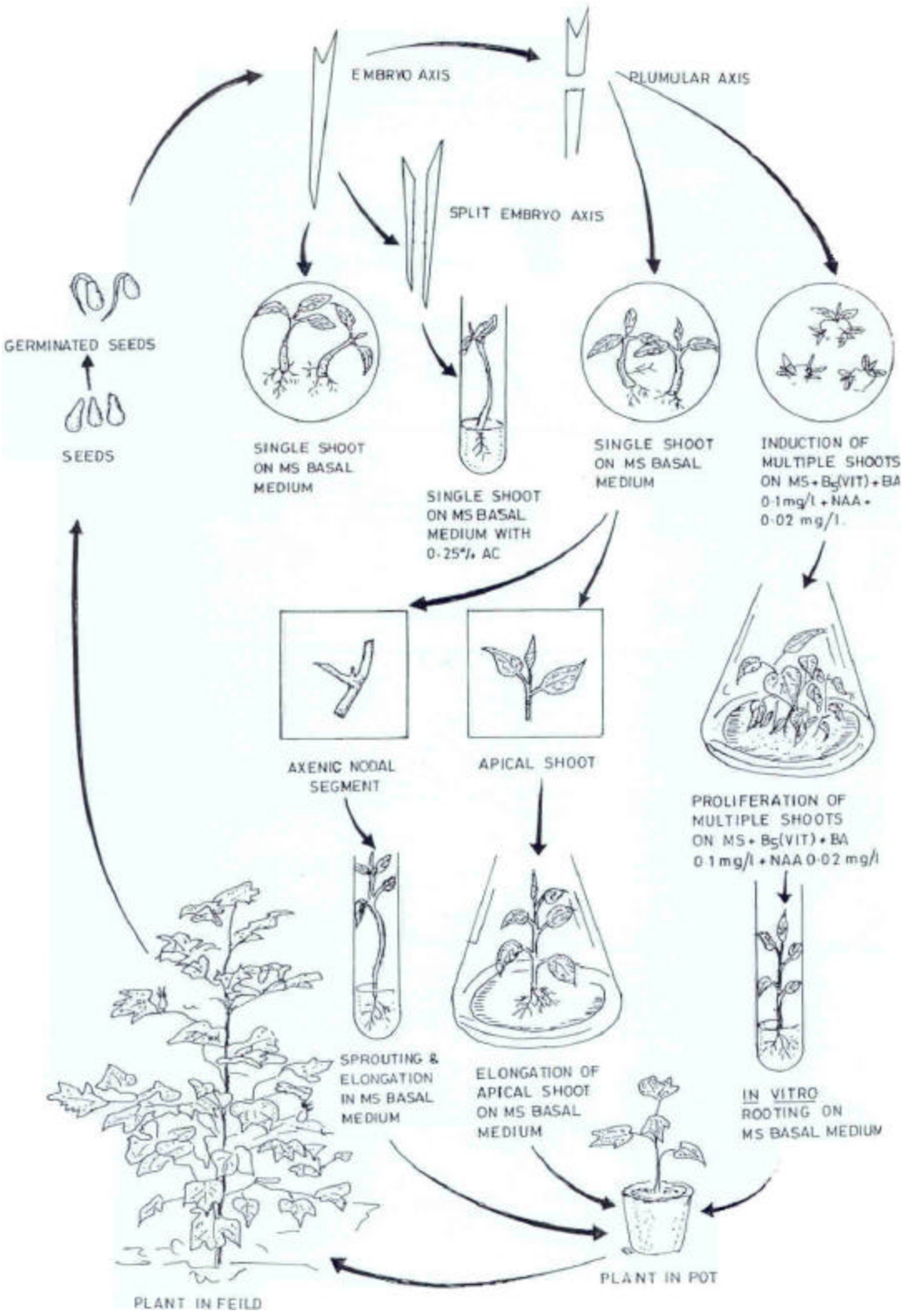
Fig. 3.8

A. Hardened tissue culture plants (derived from the multiple shoots from plumular axis explants).

B. Mature tissue culture plant of cultivar DCH-32 in field.

Fig.3.8

SCHEMATIC REPRESENTATION OF REGENERATION FROM EXPLANTS DERIVED FROM EMBRYO AXIS



3.4. Conclusion

In the present chapter, a plant regeneration protocol from intact embryo axis and split embryo axis explants of six Indian cultivars of cotton has been developed. The method is rapid and requires lesser time compared to all the methods reported earlier. Also it is simple as the additional steps of growing seedlings *in vitro* or dissection of explants under microscope are avoided. The method does not involve *de novo* regeneration pathways and is effective with all the six cultivars tested and genotype independent. The experiments on incorporation of charcoal in the medium and incubation of cultures at higher temperature are novel culture conditions. The beneficial effect of charcoal in the medium and higher incubation temperature in five out of six cultivars tested indicates synergism between these two factors for induction of higher frequency of shoots. To the best of our knowledge, this synergistic effect of charcoal with increase in incubation temperature on morphogenesis has not been reported earlier.

Plant regeneration from the axenic nodal segments has been achieved in six cultivars. The frequency of response irrespective of the cultivar was higher on the basal medium devoid of the phytohormones. Such an approach is ideal for clonal propagation of identified plants like transgenics where material for propagation is limited.

Results on plant regeneration from plumular axis explants via induction of multiple shoots have enormous significance since ours is the only report applicable to three cultivars of cotton.

The present investigations offer an opportunity to achieve plant regeneration from various explants derived from mature seed. The methods described in this chapter have application in clonal propagation of elite materials using smaller explants. Also these explants are more amenable for genetic transformation in cotton via Agrobacterium and /or particle bombardment mediated methods. Our efforts on transient gene expression in plumular axis explants involving particle bombardment mediated approach have been described in Chapter 6.

1. A revised manuscript entitled "A rapid and simple method for *in vitro* plant regeneration from split embryo axes of six cultivars of cotton" by S. Hazra, A. V. Kulkarni, A. K. Banerjee, A. B. Dhage, D. C. Agrawal, K. V. Krishnamurthy and **S. M. Nalawade** has been communicated to Biologia Plantarum
2. A manuscript entitled "Multiple shoot induction and plant regeneration from embryonic axes of Indian cultivars of cotton" by A. K. Banerjee, D. C. Agrawal, **S. M. Nalawade**, S. Hazra and K. V. Krishnamurthy has been communicated to Plant Cell Tissue and Organ Culture.
3. A part of this work has been filed as an Indian Patent 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 (Ref. No. NF-31/2000 dated 28-3-2000).

CHAPTER 4
Plant regeneration from explants consisting of pre-existing meristems from *in vitro* grown seedlings.

4.1. Introduction

Besides mature seeds (described in Chapter 3), *in vitro* grown seedling is an alternate source of explants with pre-existing meristems which can be used for clonal propagation of cotton. Such explants are already programmed for shoot formation and do not require *de novo* differentiation of meristematic tissues. Other advantages of this approach are the method is generally genotype independent and incidence of somaclonal variations occurring is minimum (Murashige 1974). A typical method of plant regeneration from explants with pre-existing meristems involves the following steps: development of single or multiple shoots from existing buds which otherwise remain dormant, elongation and proliferation of regenerated shoots, *in vitro* or *ex vitro* rooting of shoots, hardening of plantlets and their transfer to field (Ahuja 1986).

There are four published reports on clonal propagation of cotton using explants derived from *in vitro* grown seedlings (Agrawal *et al*., 1997; Hemphill *et al*., 1998; Gupta *et al*. 1997**;** Hazra *et al*. 2000). In the first report, Agrawal *et al*. (1997) could achieve plant regeneration in one Indian cotton cultivar LRK 516 using cotyledonary node explants derived from *in vitro* grown seedlings. In an another study, Hemphill *et al*., 1998 demonstrated a genotype independent procedure of plant regeneration by use of different explants derived from *in vitro* grown 28-d old seedlings. They found that plant regeneration procedure required specific concentrations of 6-benzylaminopurine (BA) depending on the origin of the meristem. Different explants appeared to possess different degrees of dormancy due to apical dominance and meristem location (Hemphill *et al*., 1998). Yet in another report, Gupta *et al*. (1997) studied regeneration potential of 3 explant types derived from *in vitro* grown seedlings of 8 cultivars of *G. hirsutum* and 2 cultivars of *G. arboreum*. They could achieve induction of multiple shoots from shoot apex along with cotyledonary node with single cotyledon attached when cultured in a medium containing BA.

These reports prompted us to optimize the medium and assess the regeneration efficiency of various explants with pre-existing meristems derived from *in vitro* grown seedlings (Hazra *et al*. 2000). In the present investigations, initially combinations of BA and NAA were tested with the cotyledonary node explant of only one cotton cultivar (NHH-44). The optimized medium was then applied to five different explant types (shoot tip, cotyledonary node, shoot apex, split cotyledonary node and petiole base) of five cultivars (NHH-44, DCH-32, DHY-286, LRK-516 and LRA-5166) to induce multiple

shoots. The explants, which demonstrated higher potential, were then cultured on a optimized medium in two types of vessels: culture tubes or conical flasks to further optimize the induction of multiple shoots. In an earlier report, Agrawal *et al.*, 1997 have demonstrated that higher number of multiple shoots can be obtained in cotton if explants were excised from seedlings grown in bottles compared to those grown in tubes. The main objective of the present chapter is:

1. to develop plant regeneration protocol from explants with pre-existing meristems derived from *in vitro* grown seedlings of five Indian cultivars of cotton.

4.2. Materials and methods

4.2.1. Plant materials

Seeds of five cotton cultivars NHH-44, DCH-32, DHY-286, LRA-5166, LRK-516 were surface sterilized and germinated on sterile moistened filter paper as described earlier in chapter 2.

4.2.2. Media and culture conditions used in the present investigations

Murashige and Skoog's (1962) salts and vitamins (hereinafter referred as MS basal medium) were used in the present set of experiments. The types of phytohormones and carbohydrate source used in different experiments are described in the relevant sections. The pH of each medium was adjusted to 5.6 - 5.8 before autoclaving. The medium was solidified with 0.58 % agar. The cultures were incubated at 30 ± 2 °C under 16 h photoperiod having light a intensity 40 µ $E/m^2/s$.

4.2.1.1. Effect of basal medium and charcoal on development of seedlings from germinated seeds.

To obtain the maximum number of seedlings development from germinated seeds, three media conditions such as (1) Full strength MS basal medium, (2) half strength MS basal medium and (3) MS basal medium with 0.25 % activated charcoal were tested. These media were supplemented with 2% sucrose and 0.58 % agar. The experiment was conducted with five cultivars. The germinated seeds (seeds with emerged radicles after 48 h of incubation in dark) were cultured in test tubes containing 20 ml of medium and incubated under conditions mentioned in section 4.2.2. The number of seedlings developed under each treatment was recorded after seven days of incubation. The experiment was repeated thrice with 20 germinated seeds in each treatment.

4.2.3. Preparation of cotyledonary node explant

In the initial experiments, cotyledonary nodes from 21-d old *in vitro* grown seedlings of cultivar NHH-44 were used as explants. These explants were obtained by removing roots, lower part of hypocotyl, cotyledons and apical shoot from the seedlings.

4.2.4. Effect of various phytohormones on induction of shoot/s from cotyledonary node explants

This initial experiment with cotyledonary node explants was carried out to assess the morphogenetic response of various phytohormones. The explants were cultured in test tubes containing 20 ml of MS basal medium supplemented with 2% sucrose, BA (0.1 –

10.0 mg/l) or kinetin (0.1 – 10.0 mg/l) or zeatin (0.1 – 5.0 mg/l) or combinations of BA (1 mg/l) and NAA (0.1 – 0.5 mg/l). The cultures were incubated under conditions as mentioned in section 4.2.2. After 3 weeks, these explants were then transferred to fresh media of similar combinations and incubated for another 3 weeks to record the number of shoots per responding explant.

4.2.5. Optimization of BA and NAA concentrations on induction of multiple shoots from cotyledonary node (CN) explants

Influence of BA and NAA on the induction of multiple shoots from cotyledonary node (CN) explants excised from 21-d old seedlings of NHH-44 was further studied. The explants were inoculated in culture tubes containing 20 ml of MS basal medium supplemented with 2% sucrose and various combinations of BA (0.5 or 1.0 mg/l) and NAA (0.05 – 0.3 mg/l). These were incubated for 3 weeks under conditions mentioned in section 4.2.2. After that, the explants were transferred to basal medium containing BA (1.0 mg/l) and 2% sucrose for two passages of 3 weeks each. At the end, the number of shoots per explant was recorded. The experiment was repeated thrice with 25 explants per treatment. The effect of different media on induction of multiple shoots was compared in a one-way analysis of variance (ANOVA). Results were subjected to student's *t* test (Steel and Torrie, 1980).

4.2.6. Effect of carbohydrate source on induction of multiple shoots from cotyledonary node explants

In this experiment, cotyledonary nodes from 21-d old seedlings of cultivar NHH-44, were cultured in test tubes containing 20 ml of induction medium (MS basal medium containing NAA 0.2 mg/l and BA 1.0 mg/l) as optimized in the previous section. The only variation was that the explants were inoculated in two sets of media: one with sucrose 2% and another with glucose 3% as carbohydrate sources. After 3 weeks, the explants from these two sets were separately transferred to media (MS basal medium with BA 1 mg/l) containing different carbohydrate sources like 2% sucrose or 3% glucose or 3% fructose or 3 % maltose separately for two subsequent passages (of 3 weeks each). The cultures were incubated under conditions mentioned in section 4.2.2. After 6 weeks, the number of shoots developed per responding explant was recorded. The experiment was repeated thrice with 20 explants per treatment. The data was analyzed for the effect of carbohydrate source on induction of multiple shoots and was compared in a

one-way analysis of variance (ANOVA). The results were subjected to the student's *t* test.

4.2.7. Optimization of BA for induction of multiple shoots from cotyledonary node explants

This experiment was carried out to optimize the BA concentration on induction of multiple shoots from cotyledonary nodes. The explants from 21-d old seedlings of cultivar NHH-44 were cultured in test tubes containing 20 ml of induction medium (MS basal medium containing 2% sucrose, BA 1.0 mg/l and NAA 0.2 mg/l) as optimized in section 3.2.6 (and hereinafter referred as MS1 medium). Explants were cultured for 3 weeks under conditions mentioned in section 4.2.2. Further these explants were transferred to media supplemented with 3% glucose (as optimized in section 4.2.6) and five concentrations of BA (0.1, 0.5, 1.0, 1.5 and 2.0 mg/l) separately. At the end of two passages (of 3 weeks each), the number of shoots developed per responding explant was scored. The experiment was repeated thrice with 20 explants per treatment. The effect of different BA concentrations on induction of multiple shoots was compared in a one-way analysis of variance (ANOVA). Results were subjected to student's *t* test.

4.2.8. Effect of seedling age on induction of multiple shoots from cotyledonary node explants

This experiment was carried out to study the influence of seedling age on the induction of multiple shoots from cotyledonary node explants of cultivar NHH-44. Explants were excised from 7, 15, 21 and 35-d old seedlings. The cotyledonary nodes from these different age seedlings were cultured in test tubes containing 20 ml of MS1 medium. After incubation for 3 weeks under conditions mentioned in section 4.2.2, the explants were transferred to MS basal medium supplemented with BA (1.0 mg/l) and 3 % glucose as optimized in previous section (and hereinafter referred as MS2 medium). The number of multiple shoots developed per responding explants was scored at the end of two passages of 3 weeks each. The experiment was repeated three times using 20 explants per treatment. The effect of seedling age on induction of multiple shoots was compared in a one-way analysis of variance (ANOVA). The results were compared using student's *t* test.

4.2.9. Effect of explant type and cultivars on induction of multiple shoots in cotton

Five different explants, cotyledonary node (CN), shoot tip (ST), shoot apex (AX), split cotyledonary node (SCN) and petiole base (PB) from 21-d old *in vitro* grown seedlings (Fig 4.1 A) were tested for their regeneration efficiency. To obtain these explants, roots, lower part of hypocotyl and cotyledons of the seedling were eliminated. The explants thus obtained consisted of an apical meristem (with or without first pair of leaves) and a cotyledonary node plus 1-1.5 cm of the hypocotyl and were referred to as a shoot tip (ST) explant. Shoot apex (AX) and cotyledonary node (CN) explants were obtained by isolating these two from the shoot tip. Cotyledonary node explants were further dissected into two halves longitudinally (SCN). Each of these two halves consisted of one axillary bud meristem and a portion of split stem. Petiole base (PB) explant with a single cotyledonary node meristem was obtained by scooping out the meristem from the side of the stem, using a fine surgical blade. Cotyledonary leaf, trimmed to 1/4 th of its original size, was retained (Fig 4.1 B). This experiment was carried out with five cotton cultivars (NHH-44, DCH-32, DHY-286, LRA-5166 and LRK-516). The five explants were cultured in test tubes on MS1 medium. These were incubated for 3 weeks under conditions mentioned in section 4.2.2. After that, the explants were transferred to MS2 medium and further incubated for two passages of 3 weeks each. At the end, the number of shoots formed per explant was recorded. The experiment was repeated three times with 20-25 explants per treatment. The data was subjected to a two-way Analysis of Variance (ANOVA) and student *t* test.

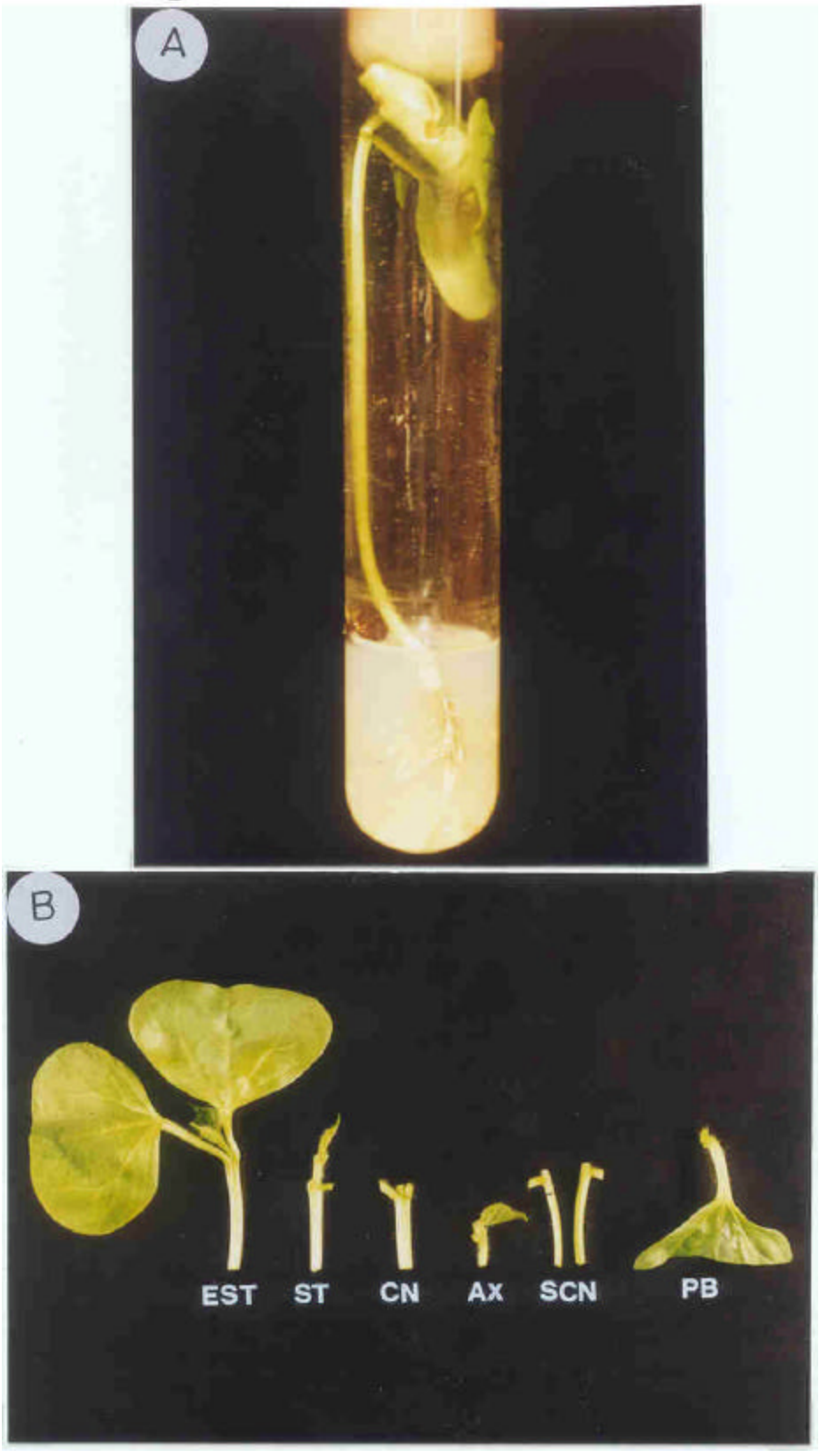
4.2.10. Effect of the culture vessel on induction of multiple shoots from split cotyledonary node and petiole base explants

The influence of culture vessel on induction of multiple shoots was assessed with split cotyledonary nodes (SCN) and petiole base (PB) explants derived from 21-d old *in vitro* grown seedlings of five cotton cultivars (NHH-44, DCH-32, DHY-286, LRA-5166 and LRK-516). The explants were cultured in test tubes containing MS1 medium and incubated under conditions mentioned in section 4.2.2 for 3 weeks. Thereafter, these explants were divided into two groups. One group was transferred to test tubes and other group to 250 ml capacity conical flasks. These vessels contained 20 and 50 ml of MS2 medium respectively. Each culture vessel had only one explant. After 3 weeks, the cultures were transferred to fresh medium correspondingly in similar vessels and

Fig. 4.1
A. *In vitro* grown seedling (21-d old).

B. Different explant types excised from *in vitro* grown seedling: EST- Entire shoot tip with the two cotyledons, ST- Shoot tip, CN- Cotyledonary node, AX- Shoot apex, SCN- Split cotyledonary nodes, PB- petiole base.

Fig. 4



incubated for further 3 weeks. At the end, the number of shoots produced from each explant in two different culture vessels was scored. The experiment was repeated four times with 20 explants per treatment. The effect of explant type, culture vessel and cultivar on induction of multiple shoot was compared in a three-way Analysis of Variance (ANOVA). The results were subjected to student's *t* test.

4.2.11. Maintenance of shoot cultures

The clusters of shoots obtained in MS2 medium in 250 ml flasks were divided into 2-3 smaller clusters using a sharp scalpel and were transferred to fresh medium in 250 ml flasks for further multiplication. The cultures were incubated under the same culture conditions and were sub-cultured every 4 weeks for maintenance. To obtain elongated shoots, the cultures were incubated for 6 weeks.

4.2.12. Rooting of *in vitro* shoots

Elongated shoots (4-5 cm long) were isolated from the clusters developed in the medium in flasks and were transferred individually to MS basal medium supplemented with 2 % sucrose and 0.58 % agar for rooting (Table 4.10). The data of rooting frequency was recorded after 4 weeks of incubation under conditions mentioned in section 4.2.2.

4.2.13. Hardening of plantlets

Fifty rooted shoots (10 rooted shoots of each cultivar) were transferred to earthen pots (8 cm) containing a mixture of sand:soil (1:1) and kept under greenhouse conditions. Hardening of the rooted shoots was done as described in chapter 2, section 2.10. Forty plants were transferred to the field after 3-4 weeks of hardening in greenhouse.

4.3. Results and Discussion

Results on development of seedlings from germinated seeds in five cultivars of cotton used in the present study; a protocol for clonal propagation using explants with pre-existing meristems of *in vitro* grown seedling; results on induction of multiple shoots from cotyledonary nodes explants following step by step standardization of various parameters like phytohormones (combinations of BA and NAA; various concentrations of BA alone), source of carbohydrate, age of the explant have been described in this chapter. Also results on application of optimized media with cotyledonary node explants to five different types of explants from *in vitro* grown seedlings of five cultivars and the influence of culture vessels (i.e. culture tubes and flasks) on induction of multiple shoots in the best responding explants (split cotyledonary node and petiole base) were also described in this chapter.

4.3.1. Effect of basal medium and charcoal on development of seedlings from germinated seeds

Murashige and Skoog's (MS) basal medium was chosen in the present study to obtain seedlings from the germinated seeds on the basis of earlier reports on cotton (Bajaj and Gill 1986; Gould *et al.* 1991; Hemphill *et al.* 1998). Among the three basal media tested, the maximum number of seedling's development occurred in full strength MS basal medium in all the five cotton cultivars. (Table 4.1). The highest percentage was recorded in cultivar NHH-44 (91.7 %) followed by DCH-32 (88.3 %), DHY-286 (83.3 %), LRK-516 (81.3 %) and LRA-5166 (80.3 %). In an earlier report, Kumar *et al.* (1998) obtained seedling development from germinated seeds by culturing on half strength MS basal medium, however they found it necessary to remove the seed coat to eliminate fungal spores. In the present study, we could get aseptic seedlings without removing the seed coat but by the stringent surface sterilization procedure as described in chapter 3.

Table 4.1: Effect of basal medium on development of seedlings from germinated seeds.

Cultivars	Seedling development (%)		
	MS	½ MS	MS+ 0.25 % Char
DCH-32	88.3 ± 2.9	75.0 ± 5.0	76.7 ± 2.9
DHY-286	83.3 ± 7.6	55.0 ± 5.0	75.0 ± 5.0
LRA-5166	80.0 ± 8.7	61.0 ± 7.6	65.0 ± 5.0
LRK-516	81.7 ± 7.6	60.0 ± 5.0	71.7 ± 5.8
NHH-44	91.7 ± 2.9	68.3 ± 7.6	73.3 ± 7.6

Data recorded after one week of incubation.
Average of 3 batches with twenty germinated seeds per treatment.

4.3.2. Effect of various phytohormones on induction of shoot/s from cotyledonary node explants

In this initial experiment, cotyledonary node explants of cultivar NHH-44 exhibited varied morphogenetic response when cultured in MS medium supplemented with various concentrations of BA, kinetin, zeatin and combinations of BA and NAA (Table 4.2). Of all the hormonal treatments tested, the maximum number of shoots (4-5 shoots) per responding explant was obtained with BA (1.0 mg/l) and NAA (0.2 mg/l). The response varied depending upon the concentration of NAA in the medium along with BA. NAA at (0.1 mg/l) and (0.5 mg/l) resulted in not only lower number of shoots but percentage of response was also lower (62.5 and 57.5 respectively). Incorporation of BA alone in the medium also resulted in varied response. BA (1-5 mg/l) induced shoots (2-3 per responding explant). Kinetin and zeatin at lower levels supported only 1-2 shoots per responding explant and did not induce multiple shoots (Table 4.2).

There are several reports available on induction of multiple shoots on incorporation of BA and NAA in the medium. Sharma *et al.* (1993) reported induction of multiple shoots in nodal segments of *Gentiana kurroo* on MS medium supplemented with

a combination of BA and NAA. In another report, axillary buds of *Asphalathus linearis* multiplied to form dense clumps of shoots in a medium formulation consisting of BA and NAA (Le Roux *et al.*, 1992).

Table 4.2: Effect of various phytohormones on induction of shoot/s from cotyledonary node explants derived from *in vitro* grown seedlings of cotton cultivar NHH-44.

Phytohormones	Conc. (mg/l)	Explant response (%)	Nature of response
BA	0.0	90.0	1-2 elongated shoots
	0.5	75.0	1-2 elongated shoots
	1.0	87.5	2-3 elongated shoots
	3.0	70.0	2-3 elongated shoots
	5.0	32.5	2-3 stunted shoots
	10.0	12.5	1-2 stunted shoots
Kinetin	0.5	82.5	1-2 elongated shoots
	1.0	75.0	1-2 elongated shoots
	2.0	80.0	1-2 stunted shoots
	5.0	67.5	1-2 stunted shoots
	10.0	12.5	One elongated shoot
Zeatin	0.1	62.5	1-2 elongated shoots
	0.5	52.5	1-2 elongated shoots
	1.0	45.0	One stunted shoot
	5.0	25.0	One stunted shoot
BA + NAA	1.0 + 0.1	62.5	2-3 elongated shoots
	1.0 + 0.2	80	4-5 elongated shoots
	1.0 + 0.5	57.5	2-3 elongated shoots

Data scored after 6 weeks of incubation.
The experiments were repeated twice with twenty explants per treatment.

4.3.3. Optimization of BA and NAA concentrations (MS1 medium) for induction of multiple shoots from cotyledonary node (CN) explants

From the previous initial experiment, it was clear that combinations of BA and NAA induced maximum number of multiple shoots from cotyledonary node explants. Therefore in the present investigations, concentrations of these two phytohormones were further optimized.

All the seven combinations of BA and NAA tested were effective in inducing multiple shoots from cotyledonary node explants of cultivar NHH-44. The percentage of response varied (20 - 80 %) depending upon the concentrations of BA and NAA in the medium (Table 4.3). Multiple shoot formation was not observed from any of the explants after first incubation for 3 weeks. In a preliminary study, it was observed that BA supported the normal growth of shoots of cotton. Therefore, in the subsequent passages all cotyledonary node explants were transferred to the basal medium containing BA (1.0 mg/l). Multiple shoots were induced in some of the explants during the second passage on this medium and the number of shoots developed from each of these explants ranged from 2-6 (Fig. 4.2). In the second passage, the merisitimatic region swelled and developed into a clonal sector. (Fig. 4.3 A-B) From this sector, prolific multiple shoots emerged in the subsequent passages (Fig. 4.3 C). The maximum shoot multiples were observed in explants inoculated on the medium containing BA (1.0 mg/l) and NAA (0.2 mg/l), followed by 2 passages in the medium containing BA (1.0 mg/l) alone. On this media sequence, explants (80%) induced on an average 6 shoots per responding explant (Table 4.3). Further multiplication and elongation of shoots in subsequent passages was obtained in the medium with BA (1.0 mg/l) alone. Multiple shoot formation was significantly higher in this media sequence compared to the others (Table 4.3). The stimulatory effect of BA on induction of multiple shoots from cotyledonary nodes has earlier been reported for cotton (Gupta *et al.* 1997), *Glycin*e (Cheng *et al.* 1980), *Pisum* (Jackson and Hobbs 1990) and *Phaseolus* (McClean and Grafton 1989). Hemplill *et al.* (1998) have also reported the use of BA for induction of multiple shoots from pre-existing meristems of primary and secondary leaf nodes and cotyledonary nodes of cotton.

Fig. 4.2

Effect of BA and NAA concentrations on induction of multiple shoots from cotyledonary node (CN) explants of 21-d old *in vitro* grown seedlings of cotton cultivar NHH-44.

Fig.4.2

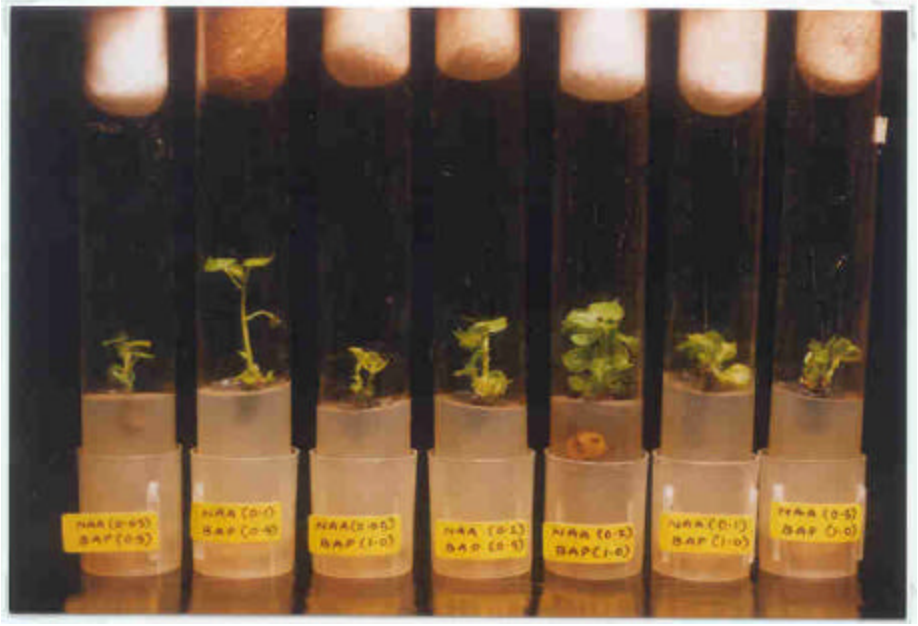


Fig. 4.3

A. - B. Formation of clonal sector in the cotyledonary node explants on BA (1 mg/l).

C. Prolific multiple shoots emerging from cotyledonary node explants on BA (1 mg/l).

Fig.4.3

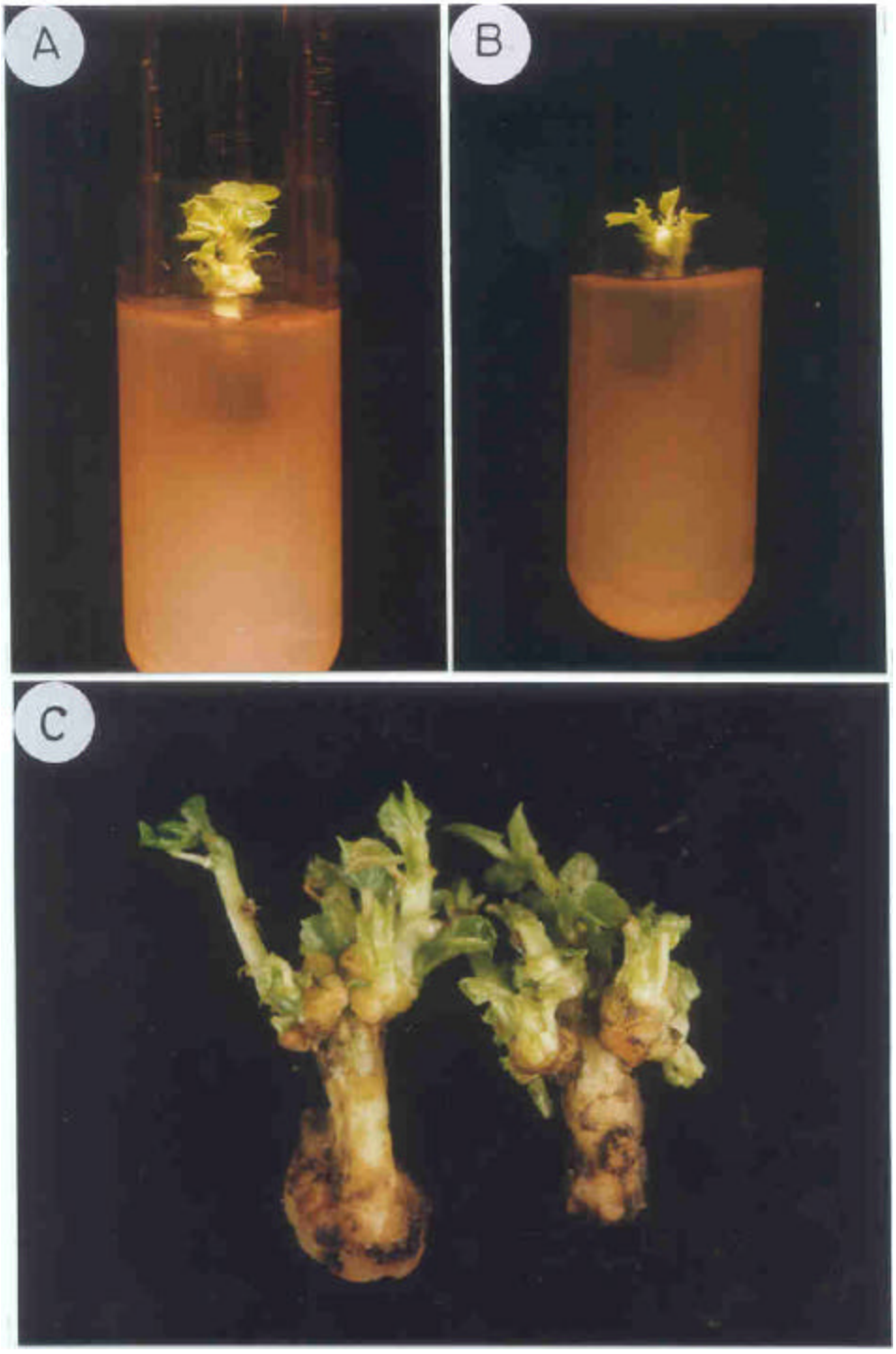


Table 4.3. Optimization of medium (MS1) for induction of multiple shoots from cotyledonary node explants of cotton cultivar NHH-44.

BA (mg/l)	NAA (mg/l)	Explant response * (%)	Avg. No. of shoots / responding explant ± sd *
0.5	0.05	58.7	2.5 ± 0.9
0.5	0.1	50.7	3.2 ± 1.0
0.5	0.2	60.0	2.7 ±0.9
1.0	0.05	53.3	3.1 ± 1.0
1.0	0.1	61.3	3.8 ± 1.1
1.0	0.2	80.0	6.0 ± 1.2**
1.0	0.3	20.0	2.3 ± 0.7

***** Average of 3 batches of each with 25 explants per treatment.
*Data scored after two passages (3 weeks each) on MS + BA (1.0 mg/l)
**Data Significant at $p \leq 0.01$

4.3.4. Effect of carbohydrate source on induction of multiple shoots from cotyledonary node explants.

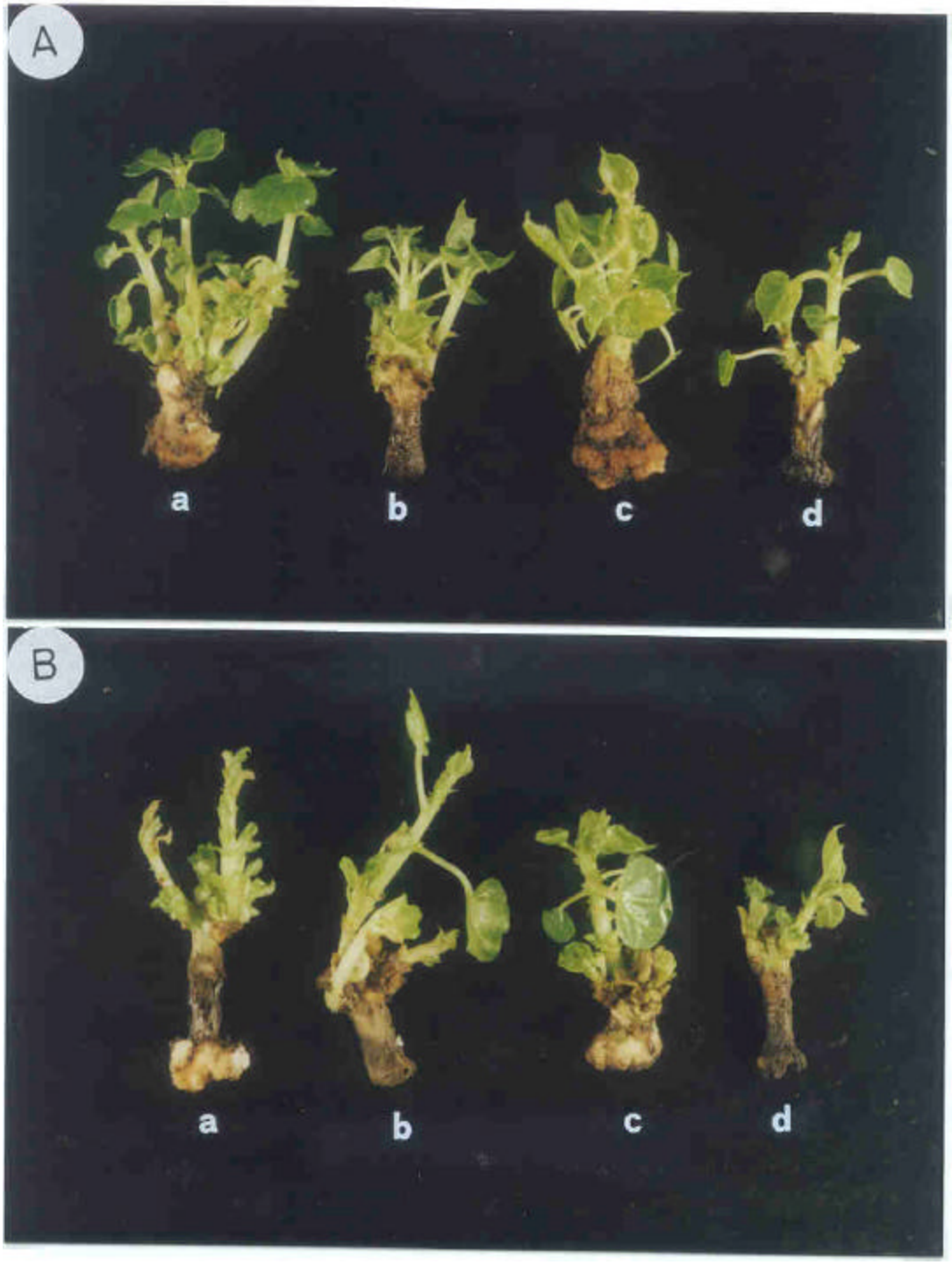
Results on the influence of carbohydrate source on induction of multiple shoots are summarized in Table 4.4. Induction of multiple shoots and the percentage of responding explants varied among the various carbohydrate sources (sucrose, glucose, fructose or maltose) tested in the sequence of two media (MS basal medium + BA 1.0 mg/l + NAA 0.2 mg/l for an initial culture of 3 weeks and thereafter MS basal medium + BA 1.0 mg/l for second and third passage of 3 weeks each) (Fig. 4.4 A-B). The observations like explant response and number of shoots per explant were recorded at the end of third passage. The maximum percentage of cotyledonary node explants responded (88.3 %) and induced multiple shoots (5.85 ± 0.10) with sucrose as a carbohydrate source in initial culture medium (MS basal + BA 1.0 mg/l + NAA 0.2 mg/l) and with glucose in the second and third passage media (MS basal medium + BA 1.0 mg/l). When sucrose was used as carbohydrate source in both media, explants (75%) developed on an average 4.02 ± 0.08 shoots. The number of shoots per responding explant decreased to 2.82 ± 0.21

Fig. 4.4

A. Effect of carbon source on induction of multiple shoots. Transfer of explants on MS1 medium with 2% sucrose to MS2 medium containing (a) 3% glucose, (b) 2% sucrose, (c) 3% fructose and (d) 3% maltose respectively.

B. Effect of carbon source on induction of multiple shoots. Transfer of explants on MS1 medium with 3% glucose to MS2 medium containing (a) 3% glucose, (b) 2% sucrose, (c) 3% fructose and (d) 3% maltose respectively.

Fig 4.4



with glucose as carbohydrate source in both the media. Use of other carbohydrate sources such as fructose or maltose in second and third passages did not increase the number of shoots per explant and was not beneficial (Table 4.4).

In conformity to our results, in majority of the published reports on cotton, either sucrose (1.5 – 2 g/l) or glucose (1.5 – 3 g/l) has been used as a carbohydrate source for induction of shoots from explants derived from seeds or seedlings (Agrawal *et al* 1997; Hemphill *et al* 1998 Gupta *et al* 1998; Gould *et al.* 1991; Zapata *et al.* 1999).

Table 4.4: Effect of carbohydrate source on induction of multiple shoots from cotyledonary nodes of cotton cultivar NHH-44.

Carbohydrate source in MS1 medium	Carbohydrate source in MS2 medium (for 2nd & 3rd passage)	Explant response (%)*	Avg. No of shoots per responding explant ± sd*
Sucrose	Glucose	88.3 ± 2.89	5.85 ± 0.10
Sucrose	Sucrose	75.0 ± 0.0	4.02 ± 0.08
Sucrose	Fructose	68.3 ± 2.89	2.73 ± 0.03
Sucrose	Maltose	55.0 ± 8.66	2.41 ± 0.16
Glucose	Glucose	85.0 ± 5.00	2.82 ± 0.21
Glucose	Sucrose	63.3 ± 2.89	2.92 ± 0.15
Glucose	Fructose	63.3 ± 5.8	2.56 ±0.12
Glucose	Maltose	56.7 ± 7.4	2.11 ± 0.23

* Average of 3 batches each with 20 explants per treatment ± sd.
*Data scored after 9 weeks [3 weeks on MS1 and 2 passages of 3 weeks each on MS with BA (1.0 mg/l)].

4.3.5. Optimization of BA (in MS2 medium) for induction of multiple shoots from cotyledonary node explants

After the standardization of the carbohydrate source, further experiments were carried out to optimize the concentration of BA in MS2 medium used in second and third passages. The response in cotyledonary node explants varied depending upon the BA concentrations in the medium. Different concentrations of BA (0.1 to 2.0 mg/l) induced shoots in range of 1-6 (Fig. 4.5 a - e). The maximum number of shoots (6.44) were

Fig. 4.5

a – e Effect of different concentrations of BA (0.1 to 2.0 mg/l) in MS2 medium on induction of multiple shoots from cotyledonary node explants. BA concentrations, a = 0.1, b = 0.5, c = 1.0, d = 1.5 and e = 2.0 mg/l.

Fig. 4.5

obtained in medium containing BA (1.0 mg/l). Concentration of BA (0.1) was the least effective and induced only 2.5 shoots per explant. The percentage of response also varied depending upon the BA concentration in the medium. The maximum percentage of response (90%) was achieved on BA (0.1 mg/l) and the minimum (56.7%) in medium without BA (controls) (Table 4.5). Thus from the present experiment, it can be concluded that BA (1.0 mg/l) is the optimum concentration for second and third passages. The regenerated shoots simultaneously elongated in the same medium.

Thus, under the present set of conditions tested, the sequence of media for induction of the optimum number of multiple shoots from cotyledonary node explants (excised from 21-d old *in vitro* grown seedlings of cultivar NHH-44) is as follows: (1) MS1= MS basal + BA (1.0 mg/l) + NAA (0.2 mg/l) + 2 % sucrose for an initial culture of 3 weeks and thereafter (2) MS2 = MS basal + BA (1.0 mg/l) + 3 % glucose for second and third passage of 3 weeks each.

Table 4.5: Effect of BA on induction of multiple shoots from cotyledonary nodes of 21-day old seedlings of cotton cultivar NHH-44.

BA (mg/l)	Explant response * (%)	Avg. No of shoots ± sd *
0.0	56.7	$1.38^{e} \pm 0.5$
0.1	90.0	$2.53^{d} \pm 0.73$
0.5	80.0	$3.33^{c} \pm 0.81$
1.0	81.7	$6.44^{a} \pm 1.02$
1.5	77.3	$4.36^{b} \pm 1.06$
2.0	58.3	$3.98^{b} \pm 1.18$

* Average of 3 batches each with 20 explants per treatment.
*Data scored after 9 weeks [3 weeks on MS1 medium and 2 passages of 3 weeks each on medium with BA (1.0 mg/l) + 3 % glucose]
Means followed by similar superscript do not differ significantly.

4.3.6. Effect of seedling age on induction of multiple shoots from cotyledonary node explants

The aim of this experiment was to optimize the seedling age for induction of multiple shoots from cotyledonary node explants of cultivar NHH-44. By using the optimized media sequence as described in section 4.3.5, the number of multiple shoots

and the percentage of response varied depending upon the seedling age (Table 4.6). Among the 7-d, 15-d, 21-d and 35-d old seedlings (Fig. 4.6 A, a - d), the axillary buds in cotyledonary node explants (Fig. 4.6 B, a - d) remained dormant and did not sprout under control conditions. The maximum number of shoots (6.4) per responding explant and the maximum percentage of response (90%) was achieved with 21-d old seedlings. The percentage of response was found to be the lowest (65%) in the explants taken from 7-d old seedlings which induced only 2.82 shoots per explant (Table 4.6) (Fig. 4.6 C, a - d).

In a similar type of study, Agrawal *et al*. 1997 had demonstrated the variation in response with different seedling age in their study on cotton cultivar LRK-516. They achieved the maximum number of shoots (4.5 per cotyledonary node explant) derived from 35-d old seedling on culture of explants on MS basal medium supplemented with BA and kinetin (2.5 mg/l each). Similarly, Gupta *et al*. (1997) have reported that the number of shoots per responding explant increased with the age of seedling (5 - 10-d old seedlings).

Table 4.6: Effect of age of seedling on multiple shoot induction from cotyledonary node of cotton cultivar NHH-44.

Seeding age	Explants responded (%)*	Avg. No. of shoots ± sd*
7 days	65.0	$2.82^{c} \pm 0.7$
15 days	76.7	$3.91^{b} \pm 1.0$
21 days	90.0	$6.38^{a} \pm 1.2$
35 days	85.0	$3.69^{b} \pm 1.0$

* Average of 3 batches each with 20 explants per treatment.
*Data scored after 9 weeks (3 weeks on MS1 medium and 2 passages of 3 weeks each on MS 2 medium)
Means followed by similar superscript do not differ significantly.

4.3.7. Effect of explant type and cultivars on induction of multiple shoots in cotton

Media sequence and seedling age optimized in previous sections were applied to five different explant types from five different cotton cultivars. Results of this elaborate experiment have been given in Table 4.7 and 4.8. In the present study, wide variations were noted in the shoot morphogenetic response among different explants from five cultivars, nine weeks after initiation of cultures from the seedling explants. In NHH-44,

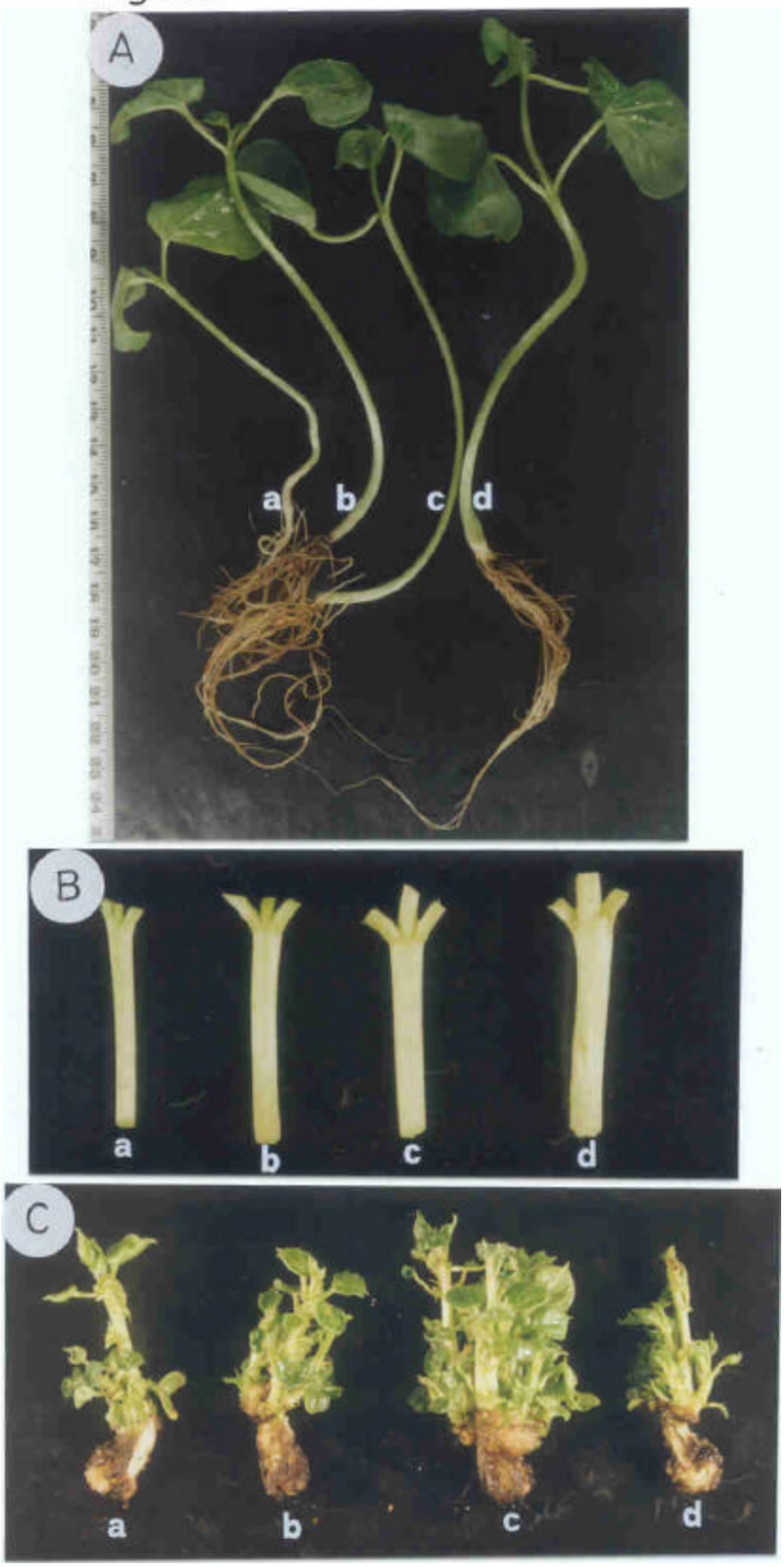
Fig. 4.6

A. *In vitro* grown seedlings. (a) 7-d old, (b) 15-d old, (c) 21-d old and (d)35-d old.

B. Cotyledonary node explants excised from (a) 7-d old, (b) 15-d old, (c) 21-d old and (d) 35-d old *in vitro* grown seedlings.

C. Induction of multiple shoots from cotyledonary node explants excised from (a) 7-d old, (b) 15-d old, (c) 21-d old and (d) 35-d old *in vitro* grown seedlings.

Fig.4.6



DCH-32, DHY-286, and LRA-5166, the cotyledonary node explants were more responsive compared to the other explants, in which responses were varied. There was no significant difference in percentage of response in all explants of LRK-516 (Table 4.7). In a separate study, Gupta *et al.* (1997), compared the morphogenetic response in eight cultivars of *G. hirsutum* and two of *G. arboreum* and found cultivar related differences in the number of shoots proliferated.

Within a cultivar, the efficiency of induction of multiple shoots varied with the explants. The shoot apex (AX) explants (0.5-0.8 cm) gave rise to single shoots or multiple shoots. However, on close examination, multiple shoot formation in AX cultures was found to arise from the axils of the newly formed true leaf primordia, giving the appearance of multiple shoots. In DCH-32, AX explants (86.17 %) responded, producing on average 3.4 shoots in each responding explant (Table 4.8). The number of shoots produced in AX explants of the other cultivars was comparatively less.

On comparison of data on multiple shoot induction from cotyledonary node explants (CN) and shoot tip explants (ST) in five cultivars, CN explants produced 3-8 shoots per responding explant compared to ST which produced lower number of shoots (3-7 shoots) (Table 4.8). The low regeneration response in ST could be due to apical dominance as reported earlier by Hemphill *et al.*, 1998; Jackson and Hobbs, 1990. Longitudinally split cotyledonary nodes (SCN) exhibited increased morphogenetic activity compared to intact cotyledonary node (CN). The split cotyledonary nodes (SCN) having a single meristem produced as many as 4-7 shoots per explant (Table 4.8), compared to 4-8 shoots per intact cotyledonary node explants having a pair of meristems. This indicates that the potential of each axillary meristem in the cotyledonary node explant (CN) is be somewhat suppressed.

Like split cotyledonary node (SCN) explants, the petiole base (PB) explant bears a single meristem but includes a smaller part of hypocotyl and 1/4th of the cotyledonary leaf. PB explant induced a large number of multiple shoots similar to SCN (Table 4.8). It was observed that the PB explant devoid of the cotyledonary leaf did not induce multiple shoots and turned brown. The morphogenetic response of the PB explant could be due to the supportive role of cotyledonary leaf (1/4th part) or due to elimination of the negative influence of the counterpart meristem. In a similar study, Gupta *et al.*, 1997 have

demonstrated the supportive role of attached cotyledon in producing multiple shoots in cotyledonary node explants of cotton.

Calculated F values for five explants (E) and five cultivars (C) were significant at $p \leq 0.01$ level, indicating cultivar and explant related differences in the morphogenetic response (ANOVA tables of Table 4.7 and 4.8).

Table 4.7: Effect of explant type and cultivars on induction of multiple shoots in cotton (% of explant response).

Explants	Explant response (%)*				
Variety ®	**NHH-44**	**DCH-32**	**DHY-286**	**LRA-5166**	**LRK-516**
AX	54.7 ± 6.1^{b} (75)	86.2 ± 6.1^{a} (75)	73.3 ± 6.1^{b} (75)	52.0 ± 6.9^{c} (75)	78.7 ± 6.1^{a} (75)
ST	65.3 ± 6.1^{b} (75)	72.0 ± 4.0^{b} (75)	78.7 ± 6.1^{ab} (65)	73.3 ± 12.5^{ab} (75)	72.0 ± 10.6^{a} (75)
CN	90.5 ± 4.2^{a} (55)	93.8 ± 2.0^{a} (80)	86.7 ± 6.1^{a} (60)	83.3 ± 7.6^{a} (60)	75.6 ± 8.0^{a} (70)
SCN	55.0 ± 13.2^{b} (60)	65.0 ± 8.7^{b} (60)	63.3 ± 2.9^{bc} (60)	61.7 ± 20.2^{bc} (60)	80.0 ± 8.9^{a} (60)
PB	76.7 ± 7.6^{b} (60)	86.0 ± 5.7^{a} (60)	78.3 ± 7.6^{ab} (60)	70.0 ± 5.0^{b} (60)	78.3 ± 7.6^{a} (60)

AX = shoot apex, ST = shoot tip, CN = cotyledonary node, SCN = split cotyledonary node, PB = petiole base explant.
*Data scored after 9 weeks (3 weeks on MS1 medium and 2 passages of 3 weeks each on on MS 2 medium).
Number in parenthesis indicates number of explants cultured.
Means followed by the same superscripts within a column do not differ significantly at $p \leq 0.05$.
ANOVA Table.

Source	d.f	SS	MSS	F–value	F-Tab 5%	F-Tab 1%
Total	74	12899.90	174.32	0.45		
Treat	24	9321.87	388.41	5.43	1.66 *	2.04 * *
E	4	1847.6	461.89	6.45	2.53 *	3.65* *
G	4	4209.1	1052.36	14.70	2.37 *	3.34 * *
E x G	16	3265.2	204.08	2.85	1.75 *	2.20 * *
Error	50	3578.03	71.56			

* Significant at $p \leq 0.05$ ** Significant at $p \leq 0.01$.
E - Explant G - Cultivar

Table 4.8: Effect of explant type and cultivars on induction of multiple shoots in cotton (Avg. No. of shoots / responding explant).

Explants ¯	Avg. No. of shoots / responding explant*				
Variety ®	**NHH-44**	**DCH-32**	**DHY-286**	**LRA-5166**	**LRK-516**
AX	1.9 ± 1.0^{c}	3.4 ± 1.0^{c}	2.4 ± 0.9^{c}	1.7 ± 0.8^{e}	2.2 ± 0.9^{c}
ST	4.0 ± 2.0^{b}	6.7 ± 1.5^{a}	4.4 ± 1.4^{a}	2.9 ± 1.1^{d}	3.1 ± 1.2^{c}
CN	6.1 ± 2.0^{a}	7.6 ± 2.8^{a}	3.5 ± 1.2^{b}	6.0 ± 1.6^{c}	4.8 ± 1.3^{b}
SCN	6.0 ± 1.5^{a}	5.9 ± 1.7^{b}	3.7 ± 1.3^{ab}	6.5 ± 1.7^{b}	6.5 ± 1.7^{a}
	(12.0)	*(11.8)*	*(7.4)*	*(12.9)*	*(13.1)*
PB	5.7 ± 1.3^{a}	6.6 ± 1.7^{ab}	3.2 ± 1.0^{b}	8.7 ± 2.4^{a}	6.2 ± 1.6^{a}
	(11.4)	*(13.6)*	*(6.4)*	*(17.4)*	*(12.3)*

AX = shoot apex, ST = shoot tip, CN = cotyledonary node, SCN = split cotyledonary node, PB = petiole base explant.
*Data scored after 9 weeks (3 weeks on MS1 medium and 2 passages of 3 weeks each on on MS 2 medium).
(Processed data, to express the potential of 2 nodes originally present in seedling.)
Means followed by the same superscripts within a column do not differ significantly at $p \leq 0.05$.

ANOVA Table

Source	d.f	SS	MSS	F	F-Tab5%	F-Tab 1%
Total	74	337.51	4.56	0.40		
Treat	24	270.39	11.27	8.39	1.5 *	1.9 **
E	4	53.28	13.32	9.92	2.52 *	3.65 **
G	4	145.11	36.28	27.03	2.37 *	3.34 **
E x G	16	72.00	4.50	3.35	1.68 *	2.2 **
Error	50	67.12	1.34			

* Significant at $p \leq 0.05$ ** Significant at $p \leq 0.01$
E - Explant G - Cultivar

4.2.8. Effect of the culture vessel on induction of multiple shoots from split cotyledonary node (SCN) and petiole base (PB) explants

From the previous experiment, it was clear that among the five explant types, split cotyledonary node (SCN) and petiole base (PB) are the best explants for induction of multiple shoots. Thus further experiments on the effect of culture vessel on induction of multiple shoots was carried out with these two explant types. A marked difference was observed in the number of multiple shoots produced from SCN and PB explants when cultured in two different culture vessels (test tubes and conical flasks), keeping the media compositions and incubation conditions unchanged (Table 4.9). Irrespective of the cultivars, explants in conical flasks produced higher number of multiple shoots compared to test tubes (Fig. 4.7 A & B). The differences in these two vessels were in the shape, volume and quantity of medium. The maximum number of shoots (19.7) were observed in the SCN explants of the cultivar LRK-516 when cultured in flasks. The lowest number of shoots (3.2) were observed in the PB explants of the cultivar DHY-286 when cultured in test tubes (Table 4.9). The calculated F value confirms the differences in the induction of multiple shoots irrespective of the cultivars and the two explants. Positive influence of larger culture vessel on induction of higher number of multiple shoots and their better growth in cotton (Agrawal *et al.*, 1997) and five woody species (Mc Clelland and Smith, 1990) has earlier been reported.

4.3.9. Maintenance of shoot cultures

During the incubation period of 6 weeks on MS2 medium, new shoot buds appeared from the base of the cluster of shoots in addition to simultaneous elongation of shoots (Fig. 4.8). As many as 7-8 shoots elongated at a time to a height of 6-8 cm in the cultures grown in 250 ml capacity conical flask. These elongated shoots were used for *in vitro* rooting experiments.

Fig. 4.7

Effect of culture vessel on induction of multiple shoots in split cotyledonary node explant (**A**) explant cultured in tube (**B**) explant cultured in 250 ml capacity conical flasks.

Fig. 4.7

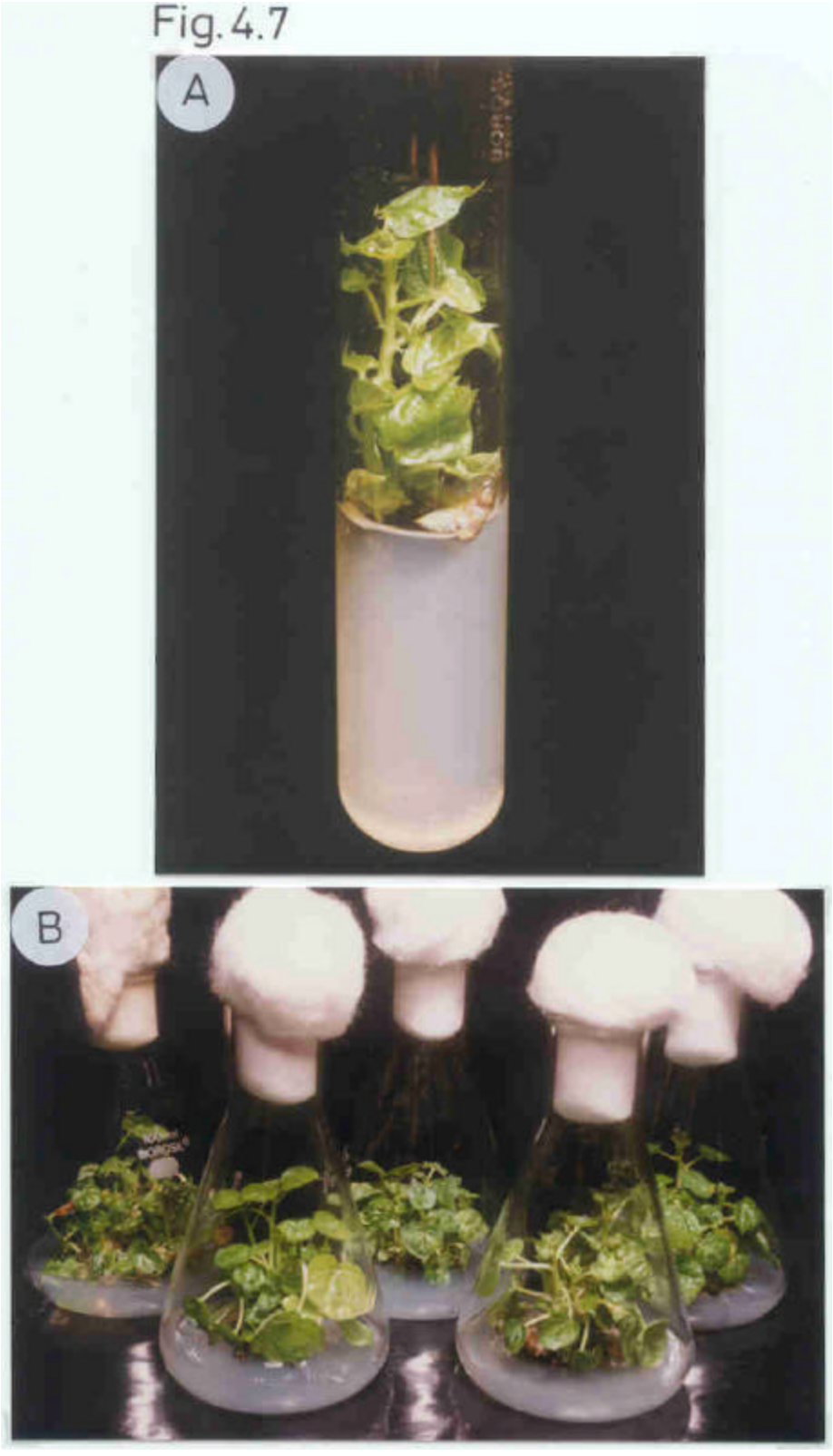


Fig. 4.8

Shoot cultures in flask.

Fig,4.8

Table 4.9: Effect of culture vessel on induction of multiple shoots from split cotyledonary node (SCN) and petiole base (PB) explants.

Cultivar	Explant type - PB Avg. No. of shoots / responding explant*		Explant type -SCN Avg. No. of shoots / responding explant*	
	Tubes	Flasks**	Tubes	Flasks**
NHH-44	5.7 ± 1.2	12.6 ± 3.3	6.0 ± 1.5	13.7 ± 3.6
DCH-32	6.6 ± 1.7	13.5 ± 3.6	5.9 ± 1.7	10.4 ± 1.9
DHY-286	3.2 ± 1.0	6.9 ± 1.1	3.8 ± 1.2	7.0 ± 1.6
LRA-5166	6.3 ± 2.1	14.4 ± 4.1	6.9 ± 1.9	17.9 ± 4.7
LRK-516	6.2 ± 1.6	13.0 ± 3.0	6.5 ± 1.9	19.7 ± 4.6

* Average of 4 batches each with 20 explants per treatment ± sd.
*Data scored after 9 weeks (3 weeks on MS1 medium and 2 passages of 3 weeks each on on MS 2 medium)
** Significant at $p \leq 0.01$

ANOVA Table

Source	Df	SS	MSS	F-Cal	F-Table	
Replicates	3	617.41	205.80	0.65	2.74	Ns
Cultivar	4	2571	514.25	1.62	2.35	Ns
Explant	1	432	432.99	1.37	3.98	Ns
Vessel	1	2793	2793	8.82	3.98	**
C x E	5	1845	369.03	1.17	2.35	Ns
C x V	5	2013	402.63	1.27	2.35	Ns
E x V	1	474	474.90	1.50	3.98	Ns
C x E x V	5	1658	331.73	1.05	2.35	Ns
Error	69	21853	316.71			
Total	95	34260	360.63			

4.3.9. Rooting of *in vitro* shoots

Initiation of roots on the elongated shoots occurred after 8-10 days of incubation on MS basal medium (Fig. 4.9 A). Rooting percentages (69.8 - 93.8 %) varied among the various cultivars tested. Secondary roots with prolific branching appeared after another 2-3 weeks. The maximum percentage of rooting (93.8 %) was observed in cultivar DCH-32 followed by DHY-286 (90.1 %), LRK-516 (79.8 %), LRA-5166 (78.3 %) and NHH-44 (69.8 %) (Table 4.10).

Table 4.10: Percentage of rooting of shoots.

Cultivar	No. of shoots tested for rooting	No. of shoots rooted	% of shoots rooted ± sd
NHH-44	36	25	69.8 ± 3.0
DCH-32	78	72	93.8 ± 5.8
DHY-286	56	51	90.1 ± 14.6
LRA-5166	48	38	78.3 ± 7.7
LRK-516	57	45	79.8 ± 7.1

Data scored after 20 days on MS medium devoid of phytohormones.

4.3.10 Hardening and survival of plantlets.

Out of the fifty rooted shoots (10 rooted shoots of each cultivar) transferred to earthen pots containing sand:soil (1:1) under greenhouse conditions, 90 % survived after 2 months (Fig. 4.9 B). On transfer to field, normal boll formation was observed in all plants after 3-4 months (Fig. 4.9 C). Similar to our earlier report (Agrawal *et al.*, 1997), there were no constraints to the survival of tissue culture raised cotton plants in soil.

(*Schematic representation of regeneration from explants derived from pre-existing meristems of in vitro grown seedlings in the following page0*).

Fig. 4.9

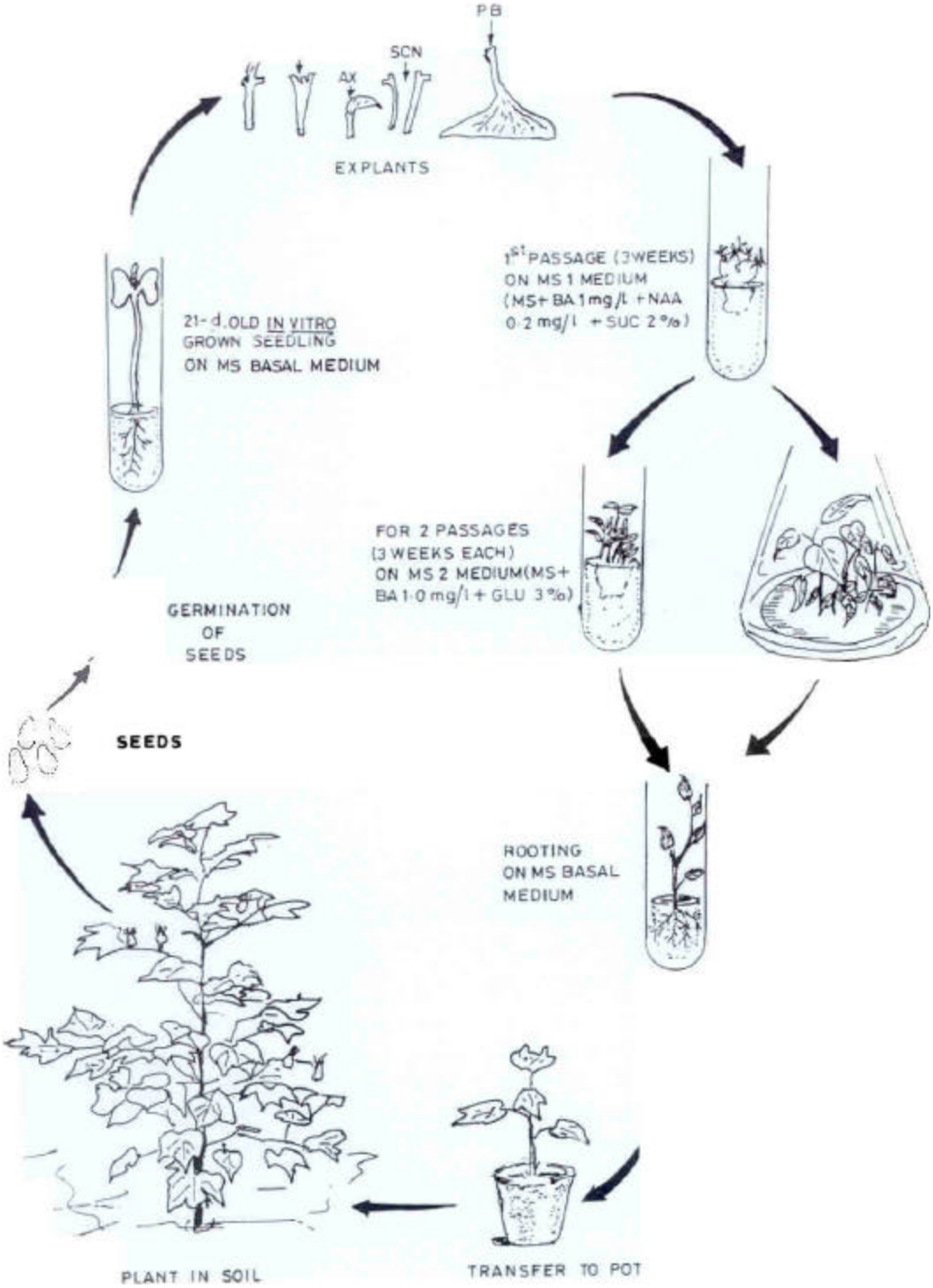
A. *In vitro* rooted shoot.

B. Hardened tissue culture plant in pot.

C. Mature tissue culture plant in field.

Fig. 4.9

SCHEMATIC REPRESENTATION OF REGENERATION FROM EXPLANTS CONSISTING OF PRE-EXISTING MERISTEMS FROM IN VITRO GROWN SEEDLINGS



4.4. Conclusion

In the present chapter, a simple and efficient method for clonal propagation using explants with pre-existing meristems derived from *in vitro* grown seedlings of five Indian cotton cultivars was standardized. The results obtained suggest that the optimized sequence of two media i.e. MS1 (MS basal medium + BA 1.0 mg/l + NAA 0.2 mg/l + sucrose 2%) for the initial culture of 3 weeks followed by MS2 (MS basal medium + BA 1.0 mg/l + glucose 3%) for the second and third passages (of 3 weeks each) were the best for the induction of multiple shoots from cotyledonary node explants derived from *in vitro* grown seedlings of cotton cultivar NHH-44. The response varied with the age of the seedling. The best response from the cotyledonary nodes was observed when the explants were excised from 21-d old seedlings. Among the different explants and cultivars tested, the morphogenetic response varied greatly with both cultivar and explant types. The increased morphogenetic response from split cotyledonary nodes compared to the intact ones is intriguing. Out of the five explant types tested, the best shoot multiplication rate was observed with split cotyledonary node and petiole base explants in all the five cultivars. The type of culture vessel also influenced the induction of multiple shoots in the two explant types tested. The larger culture vessel with more medium favored more number of shoots compared to test tubes in all the five cultivars. Like previous studies, there were no constraints to the hardening of tissue culture raised cotton plants and their survival in soil.

In nutshell, the ability to multiply, elongate and maintain the shoot cultures on a sequence of two media makes this protocol a more elegant two step method for clonal propagation of cotton using explants with pre-existing meristems. Due to an efficient multiplication rate and high frequency rooting, this method may find its application in rapid propagation of male sterile hybrid lines of cotton.

1. A manuscript entitled "Influence of the explants, genotypes and the culture vessels on sprouting and proliferation of pre-existing meristems of cotton (*Gossypium hirsutum* L. and *Gossypium arboreum* L.)" by Hazra S, Kulkarni A.V, **Nalawade S.M**, Banerjee A.K, Agrawal D.C and K.V Krishnamurthy has been published in ***In vitro* Cell and Dev. Biology Volume 36 issue 6 505-510.**
2. Sequence of two cultural media for promoting the efficiency of shoot proliferation, multiplication and elongation of shoots and a media for mass propagation of cotton plantlets from seedling explants. Hazra S, Kulkarni A.V, **Nalawade S.M**, Banerjee A.K, Agrawal D.C and K.V Krishnamurthy (**Submitted for filing an Indian Patent**)

Chapter 5
Clonal propagation using nodal segments from field-grown plants

5.1 Introduction

Use of explants (with pre-existing meristems) obtained from field grown plants is an alternate approach for clonal propagation of cotton plants. The apical meristems present in the main or lateral shoots are less differentiated and genetic ally more stable than those of other mature tissues. Thus, the plants regenerated from such meristems exhibit greater genetic stability (Kartha, 1984). The shoot meristem situated at the tip of the shoot is a dome of totipotent cells and is a center of activity for various developmental programs in the life of higher plants. The first successful meristem culture and plant formation was achieved in Nasturtium (*Tropaeolium manjus*) (Ball, 1946). Morel and Martin (1952, 1955) were first to postulate the hypothe sis that it might be possible to obtain virus-free plants by culturing the isolated meristems. Their hypothesis was based on the significant observations of Limmaset and Cornuet (1949) that viruses are not uniformly distributed in a plant and is often absent or not detectable in the apical meristem. The use of meristem for rapid clonal propagation was described by Morel with multiple protocorm formation in Cymbidium (Morel, 1960). The meristem culture is viewed as a commercially viable technology for mass propagation of numerous crop plants.

The significance of clonal propagation of mature plant lies in its use for rapid propagation of identified and/or genetically transformed cotton plants. Kaur *et. al*. 1998 obtained a large number of true to type plants using nodal segments of selected 'elite' Khair tree (*Acacia catechu* Willd.). Similarly, genetically uniform plants were obtained from nodal segments of mulbery (*Morus* Spp.) (Pattnaik and Chand, 1997) and a woody ornamental, fraser photinia (*Photinia* x *fraseri*) (Ramirez-Malagon *et. al*., 1997).

There is only one report (Bajaj and Gill, 1986) on clonal propagation of cotton using explants from field grown plants. They used excised meristems (1 mm) and shoot tips (1 cm) of *G.arboreum*, *G. hirsutum* and three embryo-derived inter-specific hybrids. The response in culture was found to be genotype dependent and the degree of success varied from 22 – 72 % depending on the genotype.

The morphology of cotton plant is rather complex because of its indeterminate growth habit. Repeated efforts by researchers over the years have yielded a great deal of literature on the anatomy and morphology of cultivated cottons (Mauney, 1984). In a growing cotton plant, there are three types of leaves: cotyledons, prophylls, and true leaves. The prophyll is inconspicuous and not ordinarily observed. An axillary meristem develops in the axil of each leaf

of the branch. This is true of a prophyll as well as the true leaves. There is no internode below the prophyll. Therefore the axillary bud of the prophyll seems to lie in the axil of the main leaf on the stem. Mauney and Ball (1959) have identified the main stem leaf axillary bud as the *first axillary* bud and the axillary of its prophyll the *second axillary* (Mauney, 1984). The prophyll axillary bud is also called "extra axillary bud" or the "accessory bud" (Mauney, 1984). We have used the same terminology in the present chapter. These accessory buds in their developmental stages are similar to that of the axillary meristem. These meristems remain dormant unless forced to elongate by pruning the branches. It is presumed (Mauney, 1984) that a plant which was forced by pruning to form new branches could develop an indefinite number of axillaries *in vivo*. Therefore, the objective of the present investigations was to induce *in vitro* multiple shoots by pruning of the primary shoot. There are no reports on clonal propagation of cotton with the axillary meristem or "extra axillary buds". In this chapter we have described the protocol of plant regeneration from the accessory buds/extra axillary bud present in the axil of nodal segments of mature field grown cotton plants of cultivar DCH-32 and NHH-44. The influence of the culture vessel on induction of multiple shoots was also studied.

5.2. Material and Methods

5.2.1. Plant material

Nodal segments of two cotton cultivars DCH-32 and NHH-44 grown in the campus field were used as explants for the present study.

5.2.1.1. Explant preparation

Twigs 18 - 24 cm in length were collected from the field-grown plants of cultivars DCH-32 and NHH-44 after flowering. Twigs were defoliated and washed thoroughly under running tap water for 2 h to wash off leaching phenolics. The nodal segments of approximately 2.5 cm each were isolated and used as explants.

5.2.1.2. Surface sterilization of the nodal segments

The nodal segments were washed with 1 % (v/v) liquid detergent (Labolene™, Qualigens, India) for 5 min. The explants were dipped in 1 % (w/v) solution of fungicide (Bavistin™, BASF, India) for 15 min with intermittent shaking. These were again washed thoroughly in running tap water and treated for 10 min with 10 % (v/v) solution of Savlon™, (NR Jet enterprises, India), a commercial disinfectant containing chlorhexidine gluconate 1.5% (v/v) and cetrimide 3% (w/v). Traces of Savlon were removed by 2 - 3 rinses with double distilled water. The explants were then surface-sterilized with 0.1 % (w/v) mercuric chloride (Qualigens, India) solution for 10 min and washed repeatedly with sterile water in aseptic conditions to remove the traces of mercuric chloride. The explants were transferred aseptically to 250 ml flasks containing 0.25 % (w/v) activated charcoal in 50 ml of sterile double distilled water and were treated for 3 h with intermittent shaking. This was followed by a mild surface sterilization with 0.1 % (w/v) mercuric chloride solution for 5 min. Traces of mercuric chloride were eliminated by repeated washing of the explants with sterile water and were spread on sterile filter papers in the laminar hood for 15 min to eliminate the surface moisture.

5.2.2. Culture medium and incubation conditions

Murashige and Skoog's (MS) medium containing mineral salts and vitamins (Murashige and Skoog, 1962) was used as the basal medium (hereinafter referred as MS basal medium). Phytohormones supplemented in the media have been described in the respective sections. All the media were supplemented with 2% sucrose and gelled with 0.65 % agar. The pH of all the media was adjusted to 5.8 before autoclaving. The cultures were incubated at 30 ± 2 °C with a 16 h photoperiod under cool fluorescent light of intensity 40 $\mu E\ m^{-2}s^{-1}$.

5.2.3. Effect of basal medium on sprouting response of the nodal segments

Initially, this experiment was carried out with only one cultivar DCH-32 to evaluate the sprouting response. Isolated nodal segments were cultured in four media compositions: (1) Full strength of MS salts + vitamins (2) Half strength of MS salts + vitamins (3) Full strength of MS + B5 vitamins and (4) Half strength of MS salts + B5 vitamins in test tubes (150 x 25 mm). The cultures were incubated for 3 weeks under conditions mentioned in section 5.2.2. After 3 weeks, the explants were transferred to fresh medium of similar compositions and incubated for another 3 weeks. At the end, the number of explants showing sprouting was scored. The experiment was repeated twice with twenty nodal segments for each treatment.

5.2.4. Effect of phytohormones on morphogenetic response of nodal segments

In a preliminary set of experiments, two phytohormones (BA and kinetin) were tested to assess the morphogenetic response of the nodal segments of cultivar DCH-32. The explants were cultured in MS basal medium supplemented with BA (0.5 – 10 mg/l) or kinetin (0.5 – 10 mg/l). After 3 weeks of incubation under conditions mentioned under section 5.2.2, the explants were transferred to fresh medium of similar combinations and further incubated for 3 weeks. At the end, observations on the morphogenetic response of cultures were recorded. The experiment was repeated twice with twenty explants for each treatment.

5.2.5. *In vitro* induction of multiple shoots in nodal segments

Nodal segments of cotton cultivar DCH-32 with axillary buds were inoculated vertically in test tubes containing 20 ml of full strength MS basal medium devoid of phytohormones. After 6 weeks of incubation under culture conditions mentioned under section 5.2.2, the number of nodal segments sprouted was recorded. This experiment was repeated three times with 258, 263 and 250 number of nodal segments respectively.

After six weeks of incubation, the single shoots, elongated from the axillary meristems of each explant, were excised and discarded. The original mother explants now devoid of the sprouted shoot and having the dormant "accessory buds" were re-cultured on MS basal medium with or with out BA (0.5 - 5.0 mg/l) singly or in combinations of BA (1 – 2 mg/l) and NAA (0.2 - 0.5mg/l). After six weeks of incubation, the number of explants forming shoots and the number of shoots developed per explant were scored. The length of elongated shoots was also measured. The shoots approximately of 1.5 cm and above were isolated and used for the rooting experiments. The mother explants were re-cultured for the second time on the fresh

medium for further proliferation and elongation of shoots. Each treatment had 20 explants. The experiment was repeated thrice and the data was analyzed statistically using ANOVA and Student's *t* test.

To test the reproducibility of the method in other cultivars, the nodal segments of cultivar NHH-44 were subjected to the same protocol used for cultivar DCH-32. Nodal segments were cultured in an agar-solidified MS basal medium. This experiment was repeated thrice with a total of 90 nodal segments. After 6 weeks of incubation the sprouted shoots were excised and discarded. The "mother explants", bearing the dormant "accessory buds", were inoculated on MS basal medium supplemented with BA 0.5 mg/l. The experiment was repeated three times with 20 replicates. Data on the number of shoots formed and elongated per responding explant was scored after 6 weeks of incubation in light. Elongated shoots were aseptically cut and used for rooting experiments and the original mother explants were re-cultured on the fresh medium for further proliferation, elongation and maintenance of shoot cultures.

5.2.6. Influence of culture vessel (test tube vs. conical flask) on induction of multiple shoots in mother explants

In the present study, the original mother explants devoid of the sprouted shoot were cultured in test tubes and 250 ml capacity conical flasks containing 20 ml and 50 ml of MS basal medium supplemented with BA 0.5 mg/l. The experiments were carried out with two cultivars NHH-44 and DCH-32. Each culture vessel had only one mother explant. The experiment was repeated thrice with 20 replicates. The response of explants and the number of shoots formed per mother explant was scored after six weeks of incubation.

5.2.6. Rooting of *in vitro* shoots

Fifty elongated shoots of cultivar DCH-32 and thirty four elongated shoots of cultivar NHH-44, obtained from the proliferation and multiplication of the accessory bud of the original mother explants were transferred to MS basal medium devoid of phytohormones and containing 2% sucrose for rooting. The cultures were incubated under the conditions mentioned in section 5.2.2. The percentage shoots rooted were recorded after 20 d.

5.2.7. Hardening of plantlets and transfer of plants to soil

Twenty five plantlets from each cultivar were transferred to earthen pots (8 cm diameter) containing sand:soil (v/v) and hardened for 3 weeks under green house conditions as

described in chapter 2 (Materials and method). Then the hardened plants were transferred to field.

5.3. Results and Discussion

This section describes the effect of the basal medium on sprouting of the meristem present in nodal segments, the morphogenetic response of the nodal segments with the phytohormones BA and kinetin. The induction and elongation of the multiple shoots from the "accessory buds" have been described. Preliminary study on the effect of the culture vessel on enhancement of the number of multiple shoots has also been described.

5.3.3. Effect of basal medium on sprouting response of nodal segments

The objective of this experiment was to optimize the basal medium so that it can be used in further experiments to induce multiple shoots from nodal segment explants (Fig 5.1 A). In the present study, the sprouting response varied among the four media tested (Table 5.1). There was heavy leaching of phenolics in all the four media tested. Therefore, after 3 weeks of incubation, it became necessary to transfer the explants to fresh media of similar compositions. The maximum sprouting response (82.5 %) was observed in full strength MS basal medium (Fig. 5.1 B) and the minimum (57.5 %) in half strength MS salts + B_5 vitamins. There was very marginal difference in percentage of response between half MS basal and full strength MS salts + B5 vitamins (Table 5.1).

Table 5.1: Effect of basal medium on sprouting of the shoot in the nodal segments of field grown plants of the cultivar DCH-32.

Media	Explant response (%)	Nature of Response
MS (salts & Vitamins)	82.5	Single elongated shoot
½ MS (salts & Vitamins)	70.0	Single elongated shoot
MS + B_5 Vitamins	72.5	Single shoot
½ MS + B_5 vitamins	57.5	Single shoot

Experiments were repeated twice with 20 explants in each treatment.
Data scored after six weeks of incubation.

5.3.3. Effect of phytohormones on morphogenetic response of nodal segments

Results on the effect of BA and kinetin on morphogenetic response of nodal segments of cultivar DCH-32 are presented in Table 5.2. The percentage response varied with the

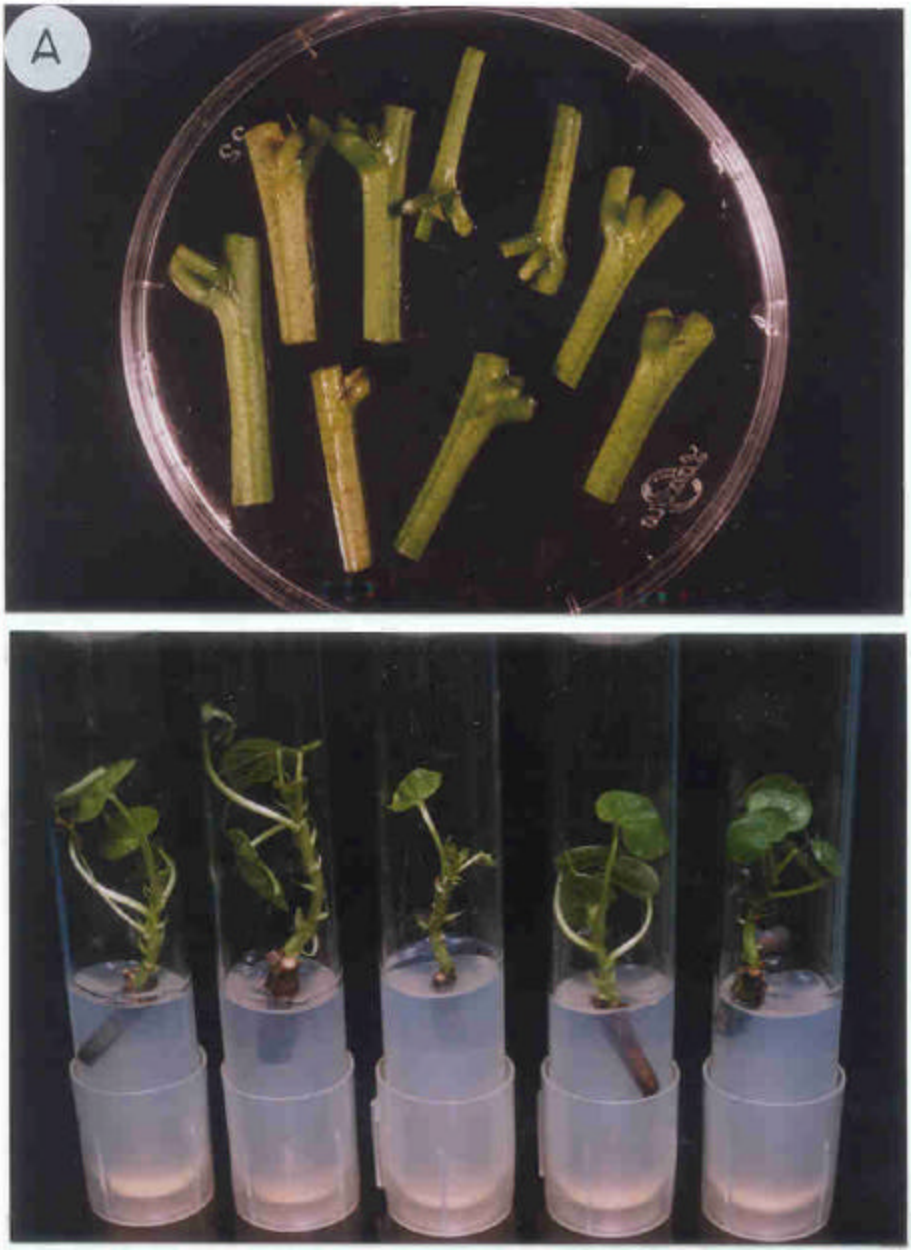
concentrations of BA and kinetin in the media. Of all the concentrations of BA and kinetin tested, BA 0.5 mg/l was the most effective and induced 2-3 shoots per

Fig. 5.1

A. Nodal segments isolated from field-grown plants.

B. Sprouting and elongation of single shoots from nodal segments after 6 weeks of culture on phytohormone-free MS basal medium.

Fig 5.



responding explant in 83.3% of the cultures. BA (1.0 –5.0 mg/l) induced only 1-2 shoots per explant (Fig. 5.2 A - E). Kinetin from 0.5 to 10 mg/l was not effective in induction of multiple shoots (Fig. 5.3 B - F). The only concentration of kinetin, which could induce more than 1 shoot in some explants, was 5.0 mg/l.

Table 5.2: Effect of phytohormones on morphogenetic response of nodal segments of cotton cultivar DCH-32.

Phytohormones	Conc. (mg/l)	Explant response (%)	Nature of response
BA	0.0	82.5	Single elongated shoot
	0.5	83.3	2-3 elongated shoots
	1.0	63.3	1-2 shoots
	2.0	77.5	1-2 shoots
	5.0	47.1	1-2 stunted shoots, Callus
	10.0	46.1	Single stunted shoot, Callus
Kinetin	0.5	58	Single elongated shoot
	1.0	45.0	Single elongated shoot
	2.0	40.5	Single shoot
	5.0	35.5	1-2 shoot
	10.0	20	Single shoot

Experiments wererepeated twice with 20 explants in each treatment.
Data scored after 6 weeks of incubation.

3.2.4. *In vitro* induction of multiple shoots from nodal segments

Since only limited number of shoots could be obtained from nodal segments with the tested concentrations of BA and kinetin in the previous experiment, we decided to test more combinations of BA and NAA for induction of multiple shoots from the explants. Also our results obtained in the previous chapter on explants derived from *in vitro* grown seedlings prompted us to test these combinations (Table 4.2 & 4.3) and remove the

Fig. 5.2

A–E. Induction of shoots from nodal segments of cultivar DCH-32 in MS basal medium with (**A**) BA 0.5 mg/l, (**B**) BA 1.0 mg/l, (**C**) 1.5 mg/l, (**D**) 2.0 mg/l and (**E**) 5.0 mg/l.

Fig. 5.2

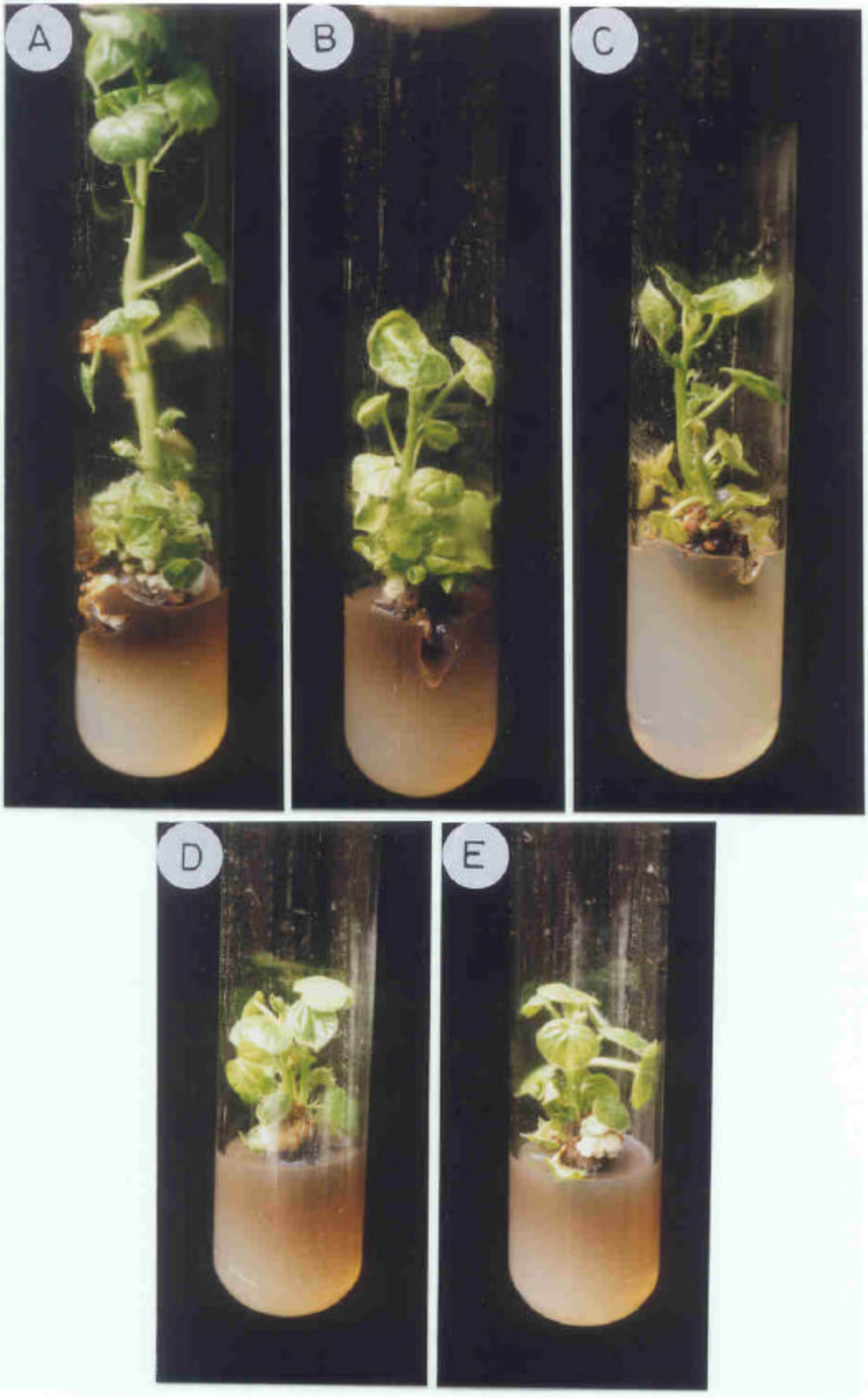


Fig. 5.3

A–F. Induction of shoots from nodal segments of cultivar DCH-32 in (**A**) MS basal medium, MS basal medium with (**B**) kinetin 0.5 mg/l, (C) kinetin 1.0 mg/l, (**D**) kinetin 2 mg/l, (**E**) kinetin 5.0 mg/l and (**F**) kinetin 10.0 mg/l.

Fig 5 3

elongated shoot from the axillary buds to overcome apical dominance and induce multiple shoots.

The sprouting of the first axillary buds from the nodal segments of cotton was affected adversely by the release of exudates (phenolics) into the medium from the cut ends of the explants. This limitation was overcome effectively by incubating the explants with 0.25% activated charcoal in water, prior to culturing in the medium. Use of charcoal to remove growth retarding phenolics released by explants in culture has been previously reported (Fridborg *et al.* 1978).

Use of nodal segments for *in vitro* propagation of plants is a routine practice (Kaur *et al.*, 1998; Ajitkumar and Seeni, 1998; Sujatha and Dhingra, 1993). In an earlier report on clonal propagation from field-grown cotton plants, development of plants occurred from cultures established from shoot tips. Shoots originated from the new axillary outgrowths after the leaves withered from the shoot tip explant (Bajaj and Gill, 1986). In the present study, nodal segments obtained from the branches of field-grown mature (flowered) plants were used as explants. As per the existing literature (Mauney, 1984) these nodes bear two meristematic buds. The first axillary buds in 82.3 % of the initial nodal explants sprouted to form shoots in MS basal medium, after incubation for 6 weeks in light. These explants produced a single shoot of approximately 2 - 4 cm in length. No multiple shoot development was observed at this stage. This step was aimed to differentiate the first axillary meristem and make it accessible for pruning (Fig. 5.4 A, a - b). The cultures were incubated for 6 weeks to get optimum sprouting of the meristem in nodal segments.

Original explants devoid of the shoot (Fig. 5.4 B) which developed from the first axillary meristem but containing the dormant accessory bud, when further cultured on MS basal medium with or without BA and NAA demonstrated varied response (Table 5.3). The positive influence of these two growth regulators on induction of meristematic activity in seedling-derived cotton explants is described in our earlier report (Hazra *et al*., 2000). The explants (46.5 %) in MS basal medium devoid of growth regulators developed single shoots. Compared to MS basal medium, incorporation of BA induced more number of shoots per responding explant (Fig. 5.5 A - E) and percentage of explant response was also significantly higher (Table 5.3). Keeping in view the positive influence of the combinations of BA and NAA (Hazra *et al*., 2000) on

seedling cultures, various formulations were (Table 4.2 & 4.3) tested for induction of multiple shoots from the

Fig. 5.4

A. (a) Elongation of primary shoot (ps) from nodal segment (ns) cultured in MS basal medium.

(b) Mother explant (m) after pruning of primary shoot (ps).

B. Mother explants obtained after pruning of primary shoots.

Fig 5 4

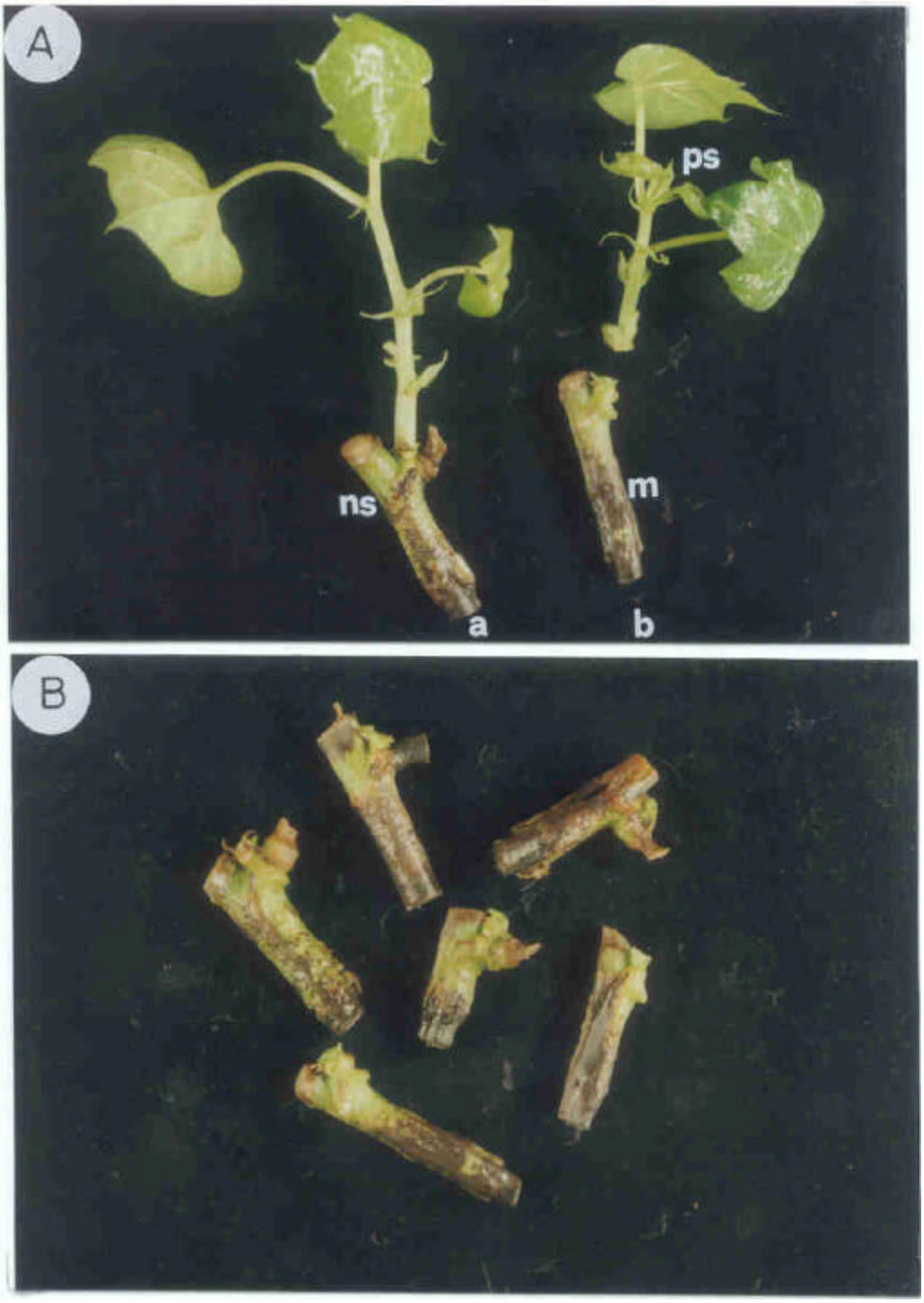
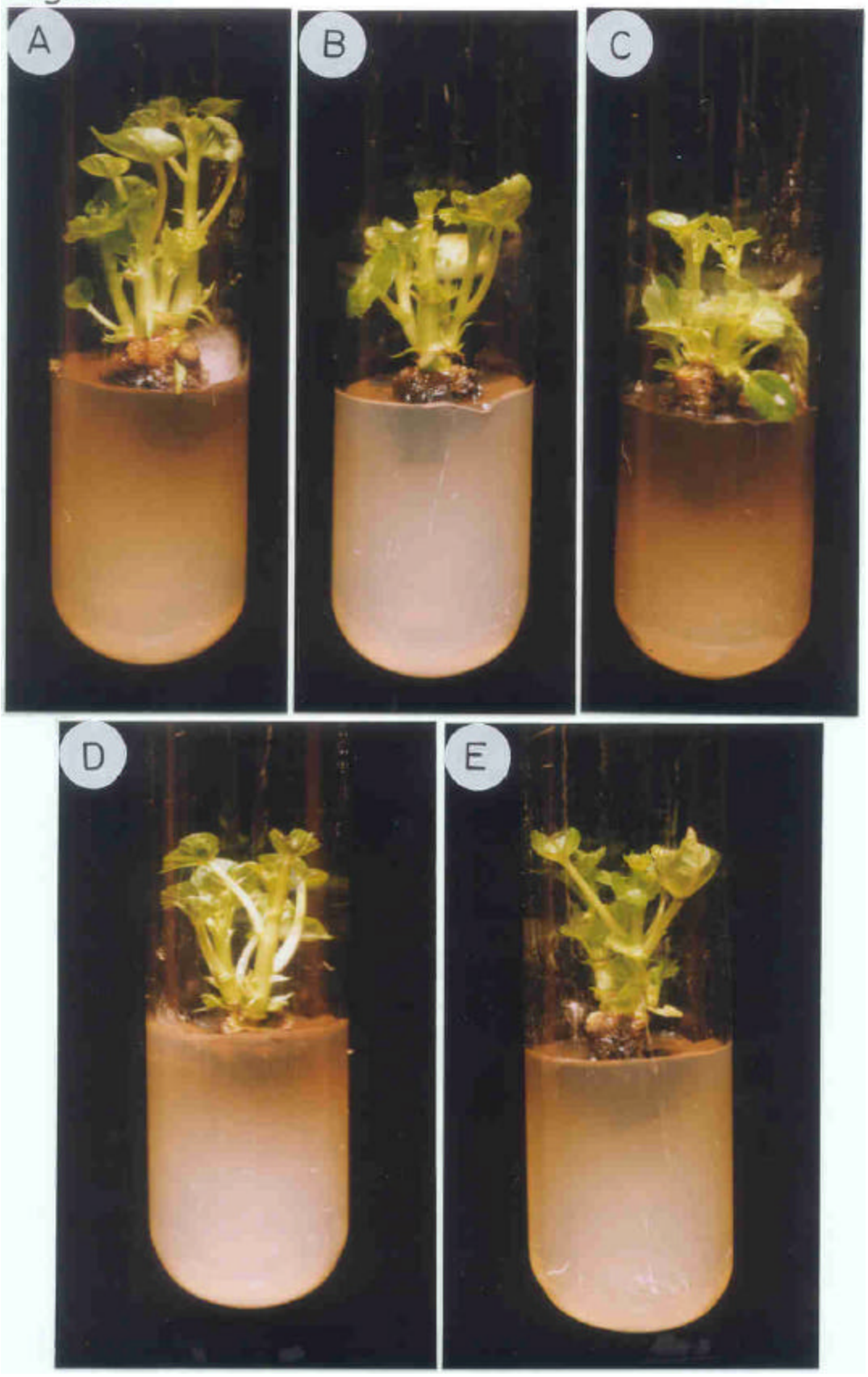


Fig. 5.5

A –E. Induction of multiple shoots from the "mother explant" (devoid of sprouted shoot) of cultivar DCH-32 on MS basal medium containing (A) BA 0.5, mg/l, (B) BA 1.0 mg/l, (C) BA 1.5mg/l, (D) BA 2.0mg/l and (E) BA 5 mg/l.

Fig.5.5



second axillary bud. The response in these treatments was higher compared to the MS basal medium devoid of growth regulators (Table 5.3). Multiple shoot induction was optimum in MS basal medium supplemented with BA 0.5 mg/l. The number of explants responding (98 %) and the number of shoots developed per explant after 6 weeks of incubation was highest (5.1 shoots per explant) in this medium. Multiple shoots were induced from the axil of the explant. The number of shoots developed from each explant ranged from 1-5. Induction of multiple buds and their differentiation to form shoots occurred in the same medium. Higher concentrations (1.0 – 5.0 mg/l) of BA were inhibitory for both sprouting and multiple shoot formation. This inhibitory effect was more pronounced in media containing BA 2 mg/l and above (Table 5.3). Compared to other cytokinins, BA is reported to be a more effective growth regulator for induction of multiple shoots from pre-existing meristems of *in vitro* grown seedlings of cotton (Hazra *et al.*, 2000; Gupta *et al*., 1997; Hemphill *et al*., 1998). The positive influence of lower levels of BA on the induction and proliferation of multiple shoots has been reported for *Salix* (Agrawal and Gebhardt, 1994) and *Morus* spp (Pattnaik and Chand, 1997).

Induction of multiple shoots by removal of apical meristems from cotyledonary node explants of seedlings has been achieved earlier in cotton (Hazra *et al*., 2000; Agrawal *et al*., 1997). The response has been attributed to enhanced branching due to removal of apical dominance (George and Sherrington, 1984). The multiple shoot development observed in the present study from the mature plant derived nodal explants devoid of sprouted shoots could be due to proliferation of a second axillary bud which is located in the axil of the prophyll. In cotton, the axillary buds pertaining to the prophyll remain dormant unless forced to elongate by pruning of the main branch (Mauney, 1984). Presumably, removal of the primary shoot and presence of a low concentration of BA stimulated the dormant axillary bud of the prophyll to form multiple growth centres, which subsequently differentiated to form multiple shoots. Inclusion of cytokinins in the medium has previously been reported to form a clonal sector in cotton meristems which causes the development of multiple shoots (Hazra *et al.*, 2000).

On an average 5.1 shoots developed from each explant cultured in MS basal medium containing BA 0.5 mg/l. Out of these, on an average 2.6 shoots (Table 5.3) elongated to more than 3 cm. The number and the length of shoots decreased with the increase of BA concentration in the medium. Media supplemented with BA 1.0, 1.5 and 2.0 mg/l supported shoot multiplication, but had limited elongation of shoots. Addition of NAA to the medium

neither supported shoot multiplication nor shoot elongation as compared to media supplemented with BA alone (Table 5.3).

Table 5.3: Effect of BA and NAA on *in vitro* induction and elongation of multiple shoots from original nodal segments (devoid of sprouted shoot) of cotton cultivar DCH-32.

BA	NAA	Explant response (%)	Avg. no of shoots/responding explant	Elongation of shoots		
				>3 cm	1.0-3.0 cm	< 1 cm
Mg/l	Mg/l	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
0.0	0.0	46.5 ± 25.6 (55)	1.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	0.7 ± 0.1
0.5	0.0	98.0 ± 3.0 (58)	5.1 ± 0.1	2.6 ± 0.1	1.4 ± 0.1	1.1 ± 0.1
1.0	0.0	94.7 ± 5.3 (56)	3.5 ± 0.4	0.0 ± 0.0	1.8 ± 0.2	1.6 ± 0.3
1.5	0.0	91.3 ±3.2 (58)	3.1 ± 0.2	0.0 ± 0.0	1.3 ± 0.2	1.8 ± 0.1
2.0	0.0	69.2 ± 7.6 (55)	2.0 ± 0.1	0.0 ± 0.0	0.6 ± 0.1	1.3 ± 0.1
5.0	0.0	74.7 ± 7.7 (55)	1.5 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	1.2 ± 0.1
1.0	0.2	75.9 ± 2.7 (58)	1.7 ± 0.1	0.0 ± 0.0	0.5 ± 0.1	1.2 ± 0.1
2.0	0.2	70.0 ± 5.0 (60)	2.1 ± 0.3	0.0 ± 0.0	0.7 ± 0.1	1.2 ± 0.5
1.0	0.5	75.0 ± 13.9 (52)	1.5 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	1.1 ± 0.2
2.0	0.5	47.5 ± 15.2 (55)	1.3 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	1.1 ± 0.1

Data scored after 6 weeks of incubation.
Average of three batches of 18-20 replicates ± SD.
The number in parenthesis indicates the total number of explants used.
The difference of response among the treatments was significant at $p \leq 0.01$ for the average number of shoots per responding explant in various media combinations tested (The least significant difference = 0.412).

The optimized protocol for DCH-32 was later tested with cultivar NHH-44 primarily to evaluate its reproducibility. On an average 77.8 % of the initial nodal explants of cultivar NHH-

44 sprouted to form a single shoot on phytohormone free MS basal medium. Multiple shoot formation was observed when the nodal explants (after pruning the shoot) were re-cultured on MS basal medium supplemented with BA 0.5 mg/l and 2 % sucrose. On an average 4.3 shoots per explant were observed in 65% of the explants, and 2 shoots per explant elongated (above 3 cm) simultaneously (Table 5.3; Fig. 5.6). This confirms the extrapolation of the method standardized with cultivar DCH-32 for induction of the second meristem from the axillary buds to proliferate and elongate after removal of the dominance of the first meristem-derived shoot to cultivar NHH-44. The morphogenetic response of the pre-existing meristems of nodal segments varied between cultivars DCH-32 and NHH-44. The nodal segments of DCH-32 were more responsive (95%) compared to NHH-44 (65%) (Table 5.3 & 5.4). It is relevant to mention here that DCH-32 is an interspecific hybrid whereas NHH-44 is an intra *hirsutum* hybrid. Both are widely grown in southwestern states of India.

Table 5.4: *In vitro* induction and elongation of multiple shoots from mother explants (devoid of sprouted shoot) of cotton cultivar NHH-44.

BA (mg/l)	Explant response (%)	Avg. no. of shoots/responding explants	Elongation of Shoots		
			>3 cm	1 – 3 cm	< 1.0 cm
0.5	65.00 ± 8.66	4.31 ± 0.16	1.87 ± 0.04	1.26 ± 0.24	1.17 ± 0.09

Data scored after six weeks of incubation.
Average of three batches of twenty replicates each ± sd.

3.2.5. Effect of culture vessel (test tubes vs flasks) on induction of multiple shoots from mother explants

In concurrence to our earlier study with plumular axis and *in vitro* grown seedling derived explants, positive influence of larger culture vessel was also observed with nodal segments (Fig. 5.7 A - B). The original mother explants devoid of sprouted shoots, when cultured in larger culture vessel (250 ml flasks) resulted in 7.1 shoots / explant in DCH-32, 6.2 in NHH-44 compared to 5.1 in DCH-32 and 4.3 shoots / explant in NHH-44 in test tubes (Table 5.5). The difference in percentage of explant response was more obvious in

cultivar NHH-44 where 95 % of explants responded in flasks compared to only 65 % in test tubes (Table 5.5).

Fig. 5.6

Induction of multiple shoots from the “mother explant” (devoid of sprouted shoot) of cultivar NHH-44 on MS basal medium supplemented with BA 0.5 mg/l.

Fig 5 6

Fig. 5.7

A-B. Effect of culture vessel: Multiple shoots from the "mother explant" (devoid of sprouted shoot) taken out from test tube (**A**) and flask (**B**) containing MS basal medium + BA 0.5 mg/l.

Fig 5 7

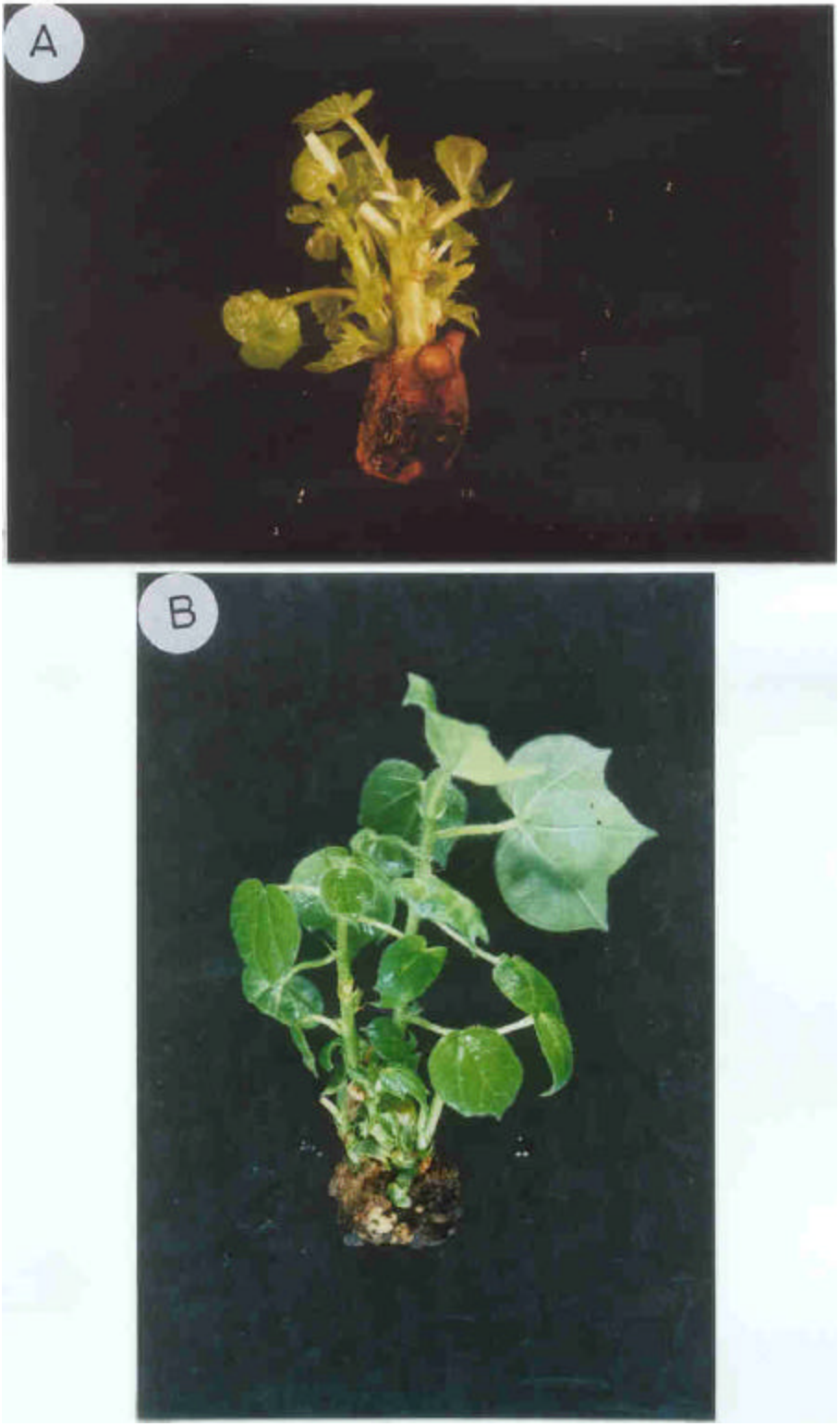


Table 5.5: Effect of culture vessel on induction of multiple shoots from mother explants of cultivars DCH-32 and NHH-44.

Cultivar ®	DCH-32		NHH-44	
Culture vessel ®	**Test Tube**	**Flask**	**Test Tube**	**Flask**
Explant response (%)	98	100	65	95
Avg. no. of shoots per Responding explants	5.1 ± 0.1	7.1 ± 1.23	4.3 ± 0.16	6.2 ± 1.34

Data scored after 6 weeks of incubation on MS basal medium with BA 0.5 mg/l.
Experiments were repeated thrice with 20 explants per treatment.

3.2.6. Rooting of *in vitro* shoots

Root initiation was observed in elongated shoots of 1.5 cm and longer after 10 d of incubation in light on phytohormone-free semi-solid MS basal medium. After 20 d of incubation root induction (Fig. 5.8 A) was achieved in 91% of the shoots of DCH-32 and 85% of the NHH-44 shoots. In conformity with the present observations, rooting of *in vitro* shoots in MS basal medium has been achieved earlier in cotton (Hazra *et al*., 2000), *Gentiana kurroo* (Sharma *et al*., 1993) and *Jatropha integrrima* (Sujatha and Dhingra, 1993).

3.2.7. Hardening of plantlets and transfer of plants to soil

Eighty-seven percent of the rooted plantlets survived following transfer to earthen pots (Fig. 5.8 B) containing a mixture of soil and sand (1:1). All plants transferred to the field showed normal growth and boll formation (Fig. 5.9).

(*Schematic representation of regeneration from nodal segments from field-grown mature plants has been given in the following page*).

Fig. 5.8

A. *In vitro* rooting of shoots on MS basal medium devoid of phytohormones.

B. Hardened tissue culture plants established in sand: soil mixture (1:1) in earthen pots kept in green house.

Fig. 5.8

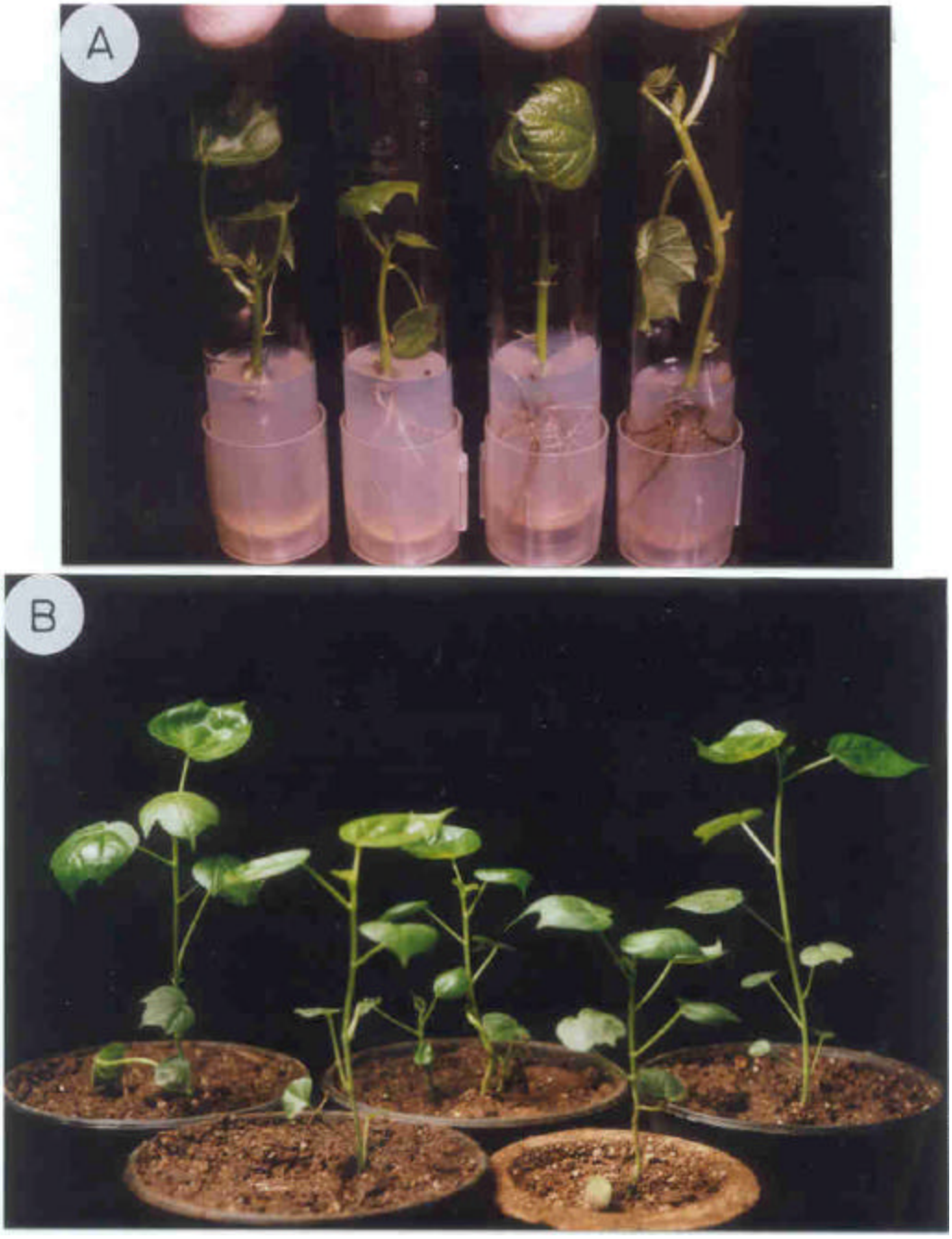
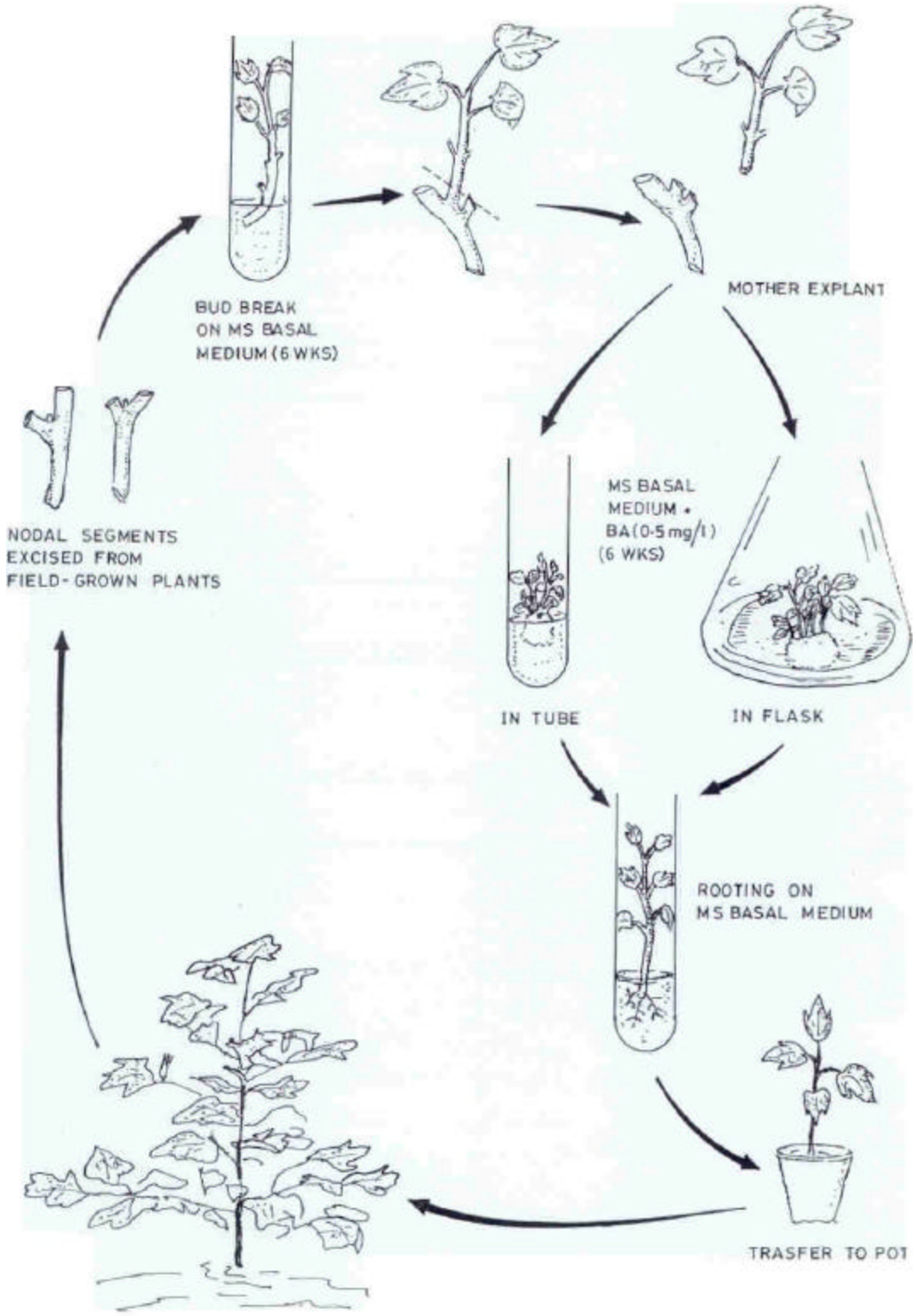


Fig. 5.9

Flowering regenerated plant in field with dehisced bolls.

Fig. 5.9

SCHEMATIC REPRESENTATION OF REGENERATION FROM NODAL SEGMENTS FROM FIELD-GROWN PLANTS



PLANT IN FEILD

5.4. Conclusion

In this present chapter, a protocol of plant regeneration from nodal segments obtained from mature field grown plants has been developed. The percentage sprouting of nodal segments varied with basal medium combination and concentration of BA or kinetin in the medium. BA was found to be most effective in induction of multiple shoots. The maximum number of multiple shoots from nodal segments of cultivar DCH-32 and NHH-44, devoid of sprouted shoot was achieved in MS basal medium with BA 0.5 mg/l. Higher concentrations of BA (1.0 - 5.0 mg/l) and incorporation of NAA did not improve induction of multiple shoots from the mother explants. The multiple shoots elongated on the same medium. *In vitro* shoots could be rooted on MS basal medium devoid of phytohormones. The hardened plants transferred to field showed normal growth and boll formation.

To the best of our knowledge, this is the first report on *in vitro* propagation of cotton using nodal segments from mature (flowered) field-grown plants. As the second crop of shoots is obtained after re-culturing the same node after pruning the initially differentiated first shoot, it appears that these shoots develop from the meristem present in the axil of the prophyll which is otherwise dormant. The method can be used for a production of large numbers of true-to-type plants after the plants are evaluated in the field at maturity for yield, fiber quality, insect resistance etc. It can also supplement breeding by reducing the period to establish stable lines and will be useful in propagation of sterile hybrids of cotton. The shoots produced by this method can be used for preservation of germplasm *in vitro* in gene banks.

A manuscript entitled "Induction of multiple shoots and plant regeneration from "accessory buds" of nodal segments from field grown mature cotton plants (*Gossypium hirsutum* L.) " by Sulekha Hazra, Dinesh C.Agrawal, Anjan K. Banerjee, Kaza V. Krishnamurthy and **Satish M. Nalawade** has been accepted in ***In vitro* Cellular and Dev. Biology Volume 37; Issue 6 (Nov/Dec) 2001 (In Press)**.

Chapter 6
Application of embryo axis explants in transformation of cotton via particle bombardment approach

6.1. Introduction

The genetic improvement of crop plants is a constant requirement for development of better quality crop. Traditional breeding programs utilize the naturally existing genetic variations by using recombinations between selected parent lines followed by progeny selection (Puonti-Kaerlas 1993). Although conventional plant breeding methods have resulted in a spectacular improvement in crop production, there are strong pressures for further improvement in crops due to explosion in population, social demands, health requirements, environmental stresses and ecological considerations (Kung 1993). Conventional plant breeding techniques have the following limitations: (1) Several back crosses are required for the establishment of new breeding lines. (2) Not all the desirable characters are naturally found (3) Dependence on the sexual compatibility of plant species (Puonti-Kaerlas 1993; Pauls 1995). These limitations have stimulated the need of more advanced technologies like genetic engineering (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. The 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

6.1.1. 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).

The first transgenic plants were developed in 1983 using a disarmed version of the Ti plasmid of *Agrobacterium tumefaciens*, a pathogenic bacterium, which can transfer part of its "T-DNA" into the plant genome (Smith 1994). *Agrobacterium*-mediated gene transfer became the method of choice due to convenience and high probability of single copy integration. The production of transgenic plants depends on the stable integration of the foreign DNA into the plant genome, followed by plant regeneration from the host cells with introduced gene/s (Walden and Wingender 1995). 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 situation changed 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).

To overcome the host range limitations of *Agrobacterium* mediated transformation, concerted efforts were made to develop alternative methods of gene transfer (Potrykus 1995). The 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)

To achieve genetic transformation in a wide range of plants, next breakthrough was the development of biolistic gun approach (Bombardment of DNA coated particles on host cells) (Klein *et al.* 1987; Sanford 1988).

6.1.2. 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 represents a biological ballistics and hence the term "biolistics" has been coined by its inventors (Sanford 1988). The technique has also been referred to as particle bombardment, microprojectile bombardment, particle acceleration, the gene gun method or the particle gun method.

6.1.3. 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 prototype 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 in 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 a ballistic approach which is particularly suitable for the controlled delivery of microprojectiles to meristem cells in situ. This method 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 delivering 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

Gene delivery through biolistic process has been demonstrated in a variety of biological systems ranging from microbes to organelles, to agriculturally important plants and to animals. Several 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. Using the biolistic method, 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

The biolistic method has several advantages over *Agrobacterium*-mediated transformation. It is a direct gene transfer method and is capable of circumventing host-range restictions of *Agrobacterium* (Klein *et al*. 1987). Plasmid construction is simplified, since DNA sequences essential for T-DNA replication and transfer in *Agrobacterium* are not required Also, false positives resulting from growth of *Agrobacterium* in host tissues are eliminated. 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 (Gray & Finer 1993). Using this method DNA can be delivered into any tissue and the

protocols are genotype independent (Walden & Wingender 1995). There is no apparent difference in the efficiency of biolistic transformation of monocotyledons vs dicotyledons (Sanford 1990).

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 to 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'.

6.1.6. Critical variables for particle bombardment

The success of plant transformations through particle bombardment depends on various variables. The 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, affect the transient gene frequency (Birch & Bower 1994). Also, the environmental factors such as 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 the 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

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

In an other 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.

Keller *et al*. (1997) developed transgenic plants resistant to bialaphos (a gene encoding phosphinothricin acetyltranferase) using Biorad-PDS/1000 He gene gun. 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 in cotton by bombarding embryogenic cell suspension cultures of cultivars Acala B1654 and Coker 315 with 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 stable expression was due to multiple bombardment of rapidly dividing cell suspension cultures and the selection of transformed cells was by gradual increase in the concentration of the antibiotic. Reports on particle bombardment mediated transformation in cotton have been given in Chapter 1 (Table 1.13).

Particle bombardment method has advantages compared to *Agrobacterium* in that it is a genotype independent technique and also any tissue can be used as an explant. To the best of our knowledge, there are no reports on transformation of Indian cultivars of cotton either by *Agrobacterium* or particle bombardment methods. In the present chapter, we have made an attempt to optimize different parameters of particle bombardment method for transient gene expression in plumular axis explants of important Indian cultivar of cotton NHH-44. Yet another objective of this study was to induce embryogenesis in the transformed callus.

6.2. Materials and Methods

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

Plasmid DNA from the *E. coli* cell line harboring p35SGUSINT was isolated by using Sigma High pure TM plasmid preparation kit (P-MINI250, Sigma, USA) and the DNA was quantified.

6.2.2. Methodology of particle bombardment

In the present study PDS-1000/He (Bio Rad Laboratories) particle delivery system was used to carry out the experiments (Fig. 6.1). Before carrying out particle bombardment, the following set up and stock solutions were prepared and kept ready:

(i) Preparation of microcarrier,

(ii) Stock solutions of spermidine and Calcium chloride ($CaCl_2$),

(iii) Sterilization of accessories of PDS-1000/He,

(iv) Precipitation of DNA onto microcarrier,

(v) Preparation of macrocarrier,

(vi) Preparation of explants.

Details of these are given in the following sections

6.2.2.1. Microcarrier preparation

Take 60 mg of gold particles (1.1 µm) in a microfuge tube.

—

Add 600 µl of 100 % ethanol to gold particles

—

Vortex on high for 2 min and centrifuge at 10,000 rpm for 1 min.

—

Remove the supernatant and repeat the step 2 & 3 thrice

—

Resuspend the particles in 1 ml of sterile distilled water and repeat the step 3

—

Remove the supernatant and repeat the step 5

—

Resuspend the microcarriers in 1 ml of sterile distilled water

—

Distribute aliquot (100 µl) of microcarrier suspension into 1.5 ml of microfuge tubes while vortexing.

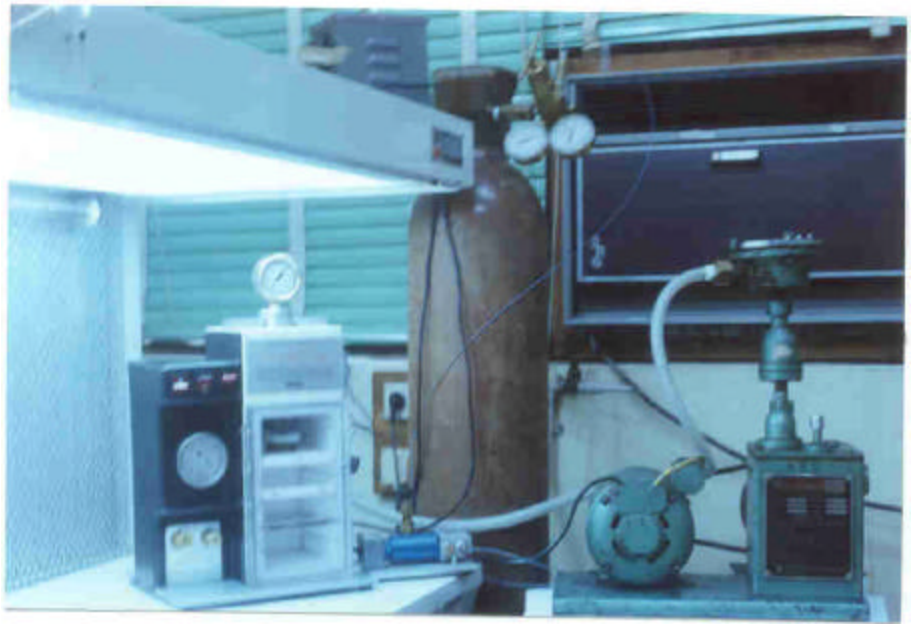
—

Store aliquots at 4 °C.

Fig. 6.1

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

Fig. 6



6.2.2.2. Preparation of 0.1 M spermidine stock

Dissolve 145 mg of spermidine (Cat. No. S 0266, Sigma, USA) in 10 ml of sterile double distilled water

—

Aliquot 40 µl of spermidine solution into 1.5 ml of microfuge tubes.

—

Store at – 20 °C.

6.2.2.3. Preparation of 2.5 M $CaCl_2$ stock

Dissolve 1.837 g of Calcium chloride ($CaCl_2$, $2H_2O$) in 5 ml of double distilled water.

—

Filter sterilize and dispense 100 µl of aliquots in 1.5 ml of microfuge tubes.

—

Store at 4° C.

6.2.2.4. 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 cleaned and 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.

6.2.2.5. Precipitation of DNA onto microcarriers

Add 10 µl of DNA (µg/µl) to 100 µl gold particles in 1.5 ml microfuge tube (as prepared in section 6.2.1.).

—

Vortex for few seconds

—

Add 100 µl of $CaCl_2$, $2H_2O$ solution and mix by vortexing

—

Add 40 µl of spermidine solution and mix by vortexing for 3 min.

—

Allow microcarriers to settle to bottom of the tube for 10 min.

—

Carefully pipette out supernatant as much as possible and discard

—

Add 200 µl of absolute ethanol (HPLC grade) to the sedimented microcarriers.

—

Vortex briefly and centrifuge at 5000 rpm for a few seconds.

—

Allow the particles to settle to bottom of the tube.

—

Pipette out supernatant carefully and discard.

—

Resuspend the particles in 120 µl of absolute ethanol.

6.2.2.6. Preparation of macrocarriers

Þ 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).

Þ 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).

6.2.2.7. Explant preparation for the bombardment

Plumular axis explants of the cultivar NHH-44 were obtained as described in section 3.2.2. of Chapter 3. Cotyledonary leaf bases were removed from plumular axis explants to expose the meristem. At about 40 explants were arranged on a sterile filter (Whatman filter paper No. 1) moistened with 1 ml of medium MS salts + Gamborg's (B_5) vitamins and 3 % glucose (hereinafter referred as MS0) 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.

6.2.2.8. Variables used for the bombardment

Plasmid p35SGUSINT, gold microcarriers (1.1µm), rupture disks (1100,1300 psi 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.

6.2.2.9. Determination of lethal dose of kanamycin for plumular axis explants

The lethal dosage (LD_{50}) of kanamycin was determined by inoculating embryo axis explants on medium MS0 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 a light intenstiy of 40 µ mol $m^{-2}s^{-1}$.

6.2.2.10. Culture of plumular axis explants after bombardment

After bombardment, the explants were kept in dark for 24 h. Thereafter, these were transferred to a semi-solid MS1 or MS2 medium (describe in section 6.2.2.11)

containing kanamycin (50 mg/l) and were incubated at 30 ^{0} C at a light intensity of 40 µ mol $m^{-2}s^{-1}$.

6.2.2.11. Culture medium for the bombarded plumular axis explants

The bombarded explants were randomly cultured in the following two sets of media for plant regeneration and callus initiation:

1. ***Culture medium for plant regeneration***: MS salts + Gamborg's (B_5) vitamins + BA (0.1 mg/l) + NAA (0.02 mg/l) and 2 % sucrose. (herein after referred as MS1 medium, standardized medium for induction of multiple shoots from plumular axis explants)

Leaf bits from the regenerated shoots surviving for 3 months on this selection medium were tested for GUS assay.

2. ***Culture medium for callus initiation***: MS salts + Gamborg's (B_5) vitamins supplemented with TDZ (0.1) + NAA (0.01mg/l) + 3 % glucose (hereinafter referred as MS2 medium).

The induced callus on this selection medium after 4 weeks of incubation was tested for the GUS assay. The callus was further incubated for 8 weeks under the conditions mentioned in section 2.2.2.10. This 12 weeks old GUS positive callus was further used to study the effect of various phytohormones on morphogenetic responses (as described in section 6.2.2.13). Both the media were solidified with 0.65% agar (Hi-media, India).

6.2.2.12. Transient expression in plumular axis explants (GUS assay)

Histochemical analysis was carried out to determine the β-glucoronidase activity in embryo axis. Random samples of plumular axes, after 72 h of incubation were immersed in 1mM X-Gluc solution in microtiter multiwell plates ("Sigma chemical CO.", USA) and incubated overnight at 37 °C for 24 h (Stomp 1992). The tissues were then bleached in 100 % ethanol before observation. Blue loci, indicative of transient GUS expression were counted under a sterioscopic microscope. Details of the GUS solution (reagent mix) are given in Table 6. 1.

Table 6.1: 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

6.2.2.13. Effect of various phytohormones on morphogenetic response in GUS positive calli

Pieces of GUS positive calli (100 mg each) were inoculated in 250 ml capacity conical flasks containing 50 ml of MS0 (MS salts + B_5 Vitamins + 3 % glucose) medium supplemented with the phytohormone. Various phytohormones like 2,4-D (0.1- 10.0 mg/l), 2,4,5-T (0.1- 10.0 mg/l), NAA (0.01- 10.0 mg/l), picloram (0.01-10.0 mg/l) and TDZ (0.01- 10.0 mg/l) in different concentrations were tested for induction of somatic embryogenesis. The flasks were shaken (150 rpm) on a rotary shaker at 25 ± 2 °C temperature under a light intensity of 40 µ mol $m^{-2}s^{-1}$. This experiment was done once with three replicates of each treatment. The observations were recorded after 1 month of incubation. The friable green callus (100 – 150 mg) obtained in TDZ (0.1-1.0 mg/l) were re-cultured on the fresh medium of similar combinations at one month intervals for 6 months.

For development of further stages, the structures resembling early stages of somatic embryos were transferred to various media combination as described in Table 6.7. Observations were taken after 4 weeks of incubation.

Histochemical analysis was carried out to determine the β-glucoronidase activity in GUS positive callus and the structures obtained from the callus. Small pieces of calli and the intact structures were 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.

6.3. Results and Discussion

This section describes the transient gene expression in the plumular axis explants of cotton cultivar NHH-44 through particle bombardment mediated transformation method. The experiments carried out to study the morphogenetic response in a GUS positive callus line have also been described.

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

The yield of plasmid DNA (p35SGUSINT) (Fig. 6.2) 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 for plumular axis explants

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. Plumular 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. Hence, kanamycin at 50 mg/l was taken as the selection pressure for the explants bombarded with plasmid p35GUSINT.

6.3.6. Transient gene expression in plumular axis explants (Gus assay)

Transient gene expression was achieved in the plumular axis explants of the cotton cultivar NHH-44 (Fig. 6.3 A-D). 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.

Plumular axis explants when bombarded with gold microcarriers coated with plasmid p35SGUSINT using rupture disc (1100 psi) and a target distance of 6 cm, the GUS expression was observed in 27.1 % of explants, while 22.6 % of explants showed GUS expression when bombarded with 1300 psi rupture disk and 8 cm target distance (Table 6.2).

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

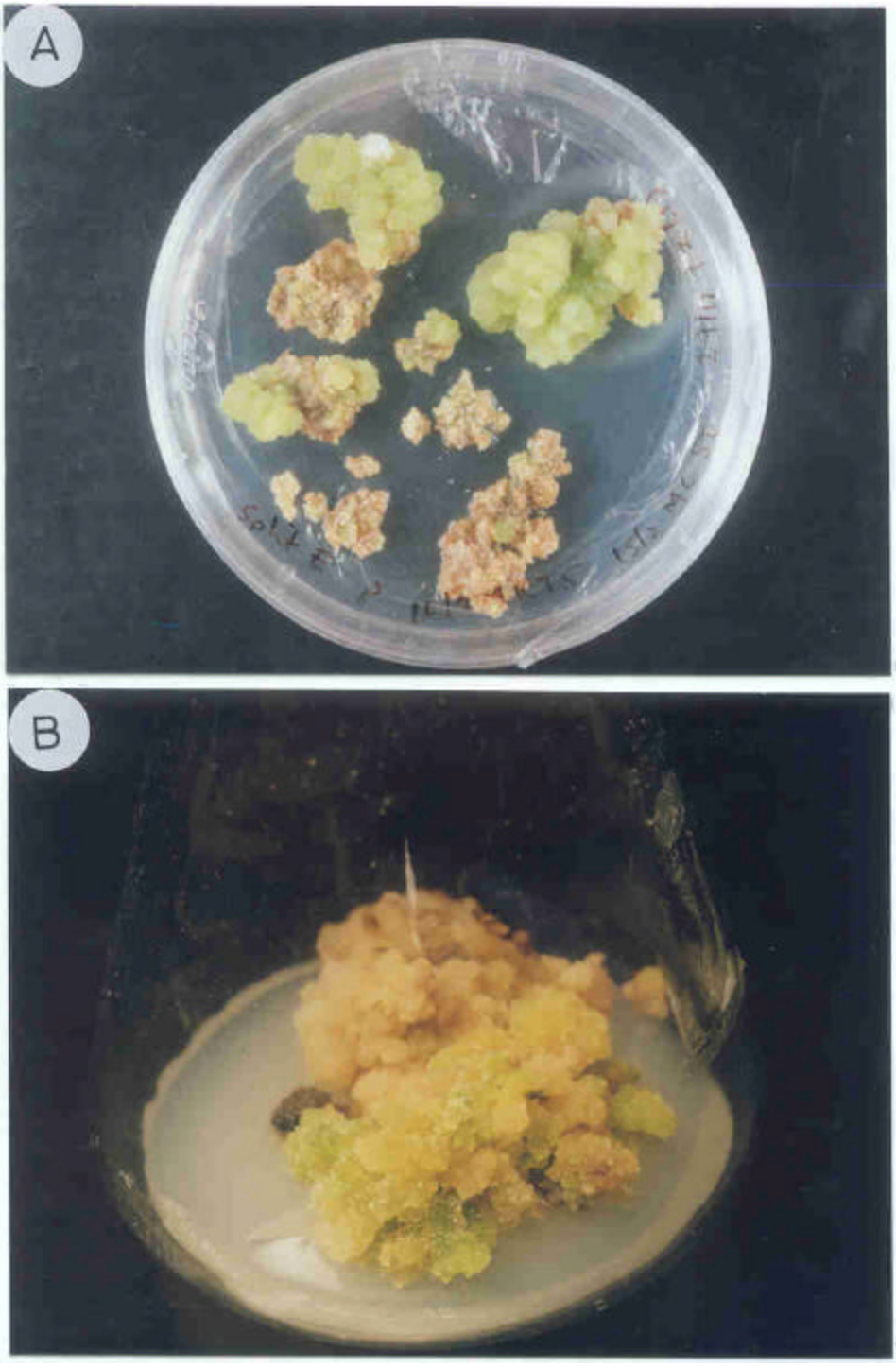
Fig. 6.2

Plasmid map of p35SGUSINT

Fig. 6.3

A-D. GUS expression in different regions of plumular axes bombarded with gold particles coated with plasmid p35SGUSINT.

Fig 6 4



established. It is because transient expression of introduced gene can be studied only 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).

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). 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.

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, plumular 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 have 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 6.2: Transient GUS expression in plumular axes after bombardment.

Cultivar – NHH-44

Explant – plumular axis (2 mm)

Plasmid – p35 GUS INT

Microcarrier – Gold

Rupture disc – 1100psi / 1300 psi

No. of Explants bombarded	Rupture disc (psi)	Target distance (cm)	No. of explants tested for GUS*	No. of explants showing GUS	% of explants showing GUS expression
960	1300	8	31	7	22.6
1250	1100	6	48	13	27.1

* Explants tested after 72 h.

6.3.7. Culture of plumular axis explants after bombardment

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).

Of the 650 explants (Out of the total 960 explants bombarded with rupture disc of 1100 psi and target distance of 6 cm) cultured on MS1 medium, 78.8 % developed single shoots in 2 weeks (Table 6.3). Of these only five survived on kanamycin selection medium (50 mg/l) after 3 months. Leaf samples from these shoots were found to be GUS negative. However, a marginally lower number of explants (73.3 %) developed single shoots when bombarded with rupture disc 1300 psi and 8 cm target distance. None of the shoots survived on the medium containing kanamycin selection pressure (50 mg/l) (Table 6.3).

Shoots which survived on selection pressure 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 single shoots in plumular axis explants after bombardment.

Cultivar – NHH-44

Explant – plumular axis (2 mm)

Plasmid – p35 GUS INT

Microcarrier – Gold

Rupture disc – 1100psi / 1300 psi

Culture medium – MS basal medium + B5 vit + BA 0.1 mg/l + NAA 0.02 mg/l

Number of explants bombarded	Rupture disc (psi)	No. of explants developed single shoots after 2 weeks	Percentage of axes developed single shoots after 2 weeks	Number of shoots survived on kanamycin up to 12 wks
530	1300	390	73.6	-
650	1100	512	78.8	5

In another set of experiments, callus initiation in 4 out of 600 explants (bombarded with 1100 psi rupture disc and target distance 6 cm) was observed when cultured on the MS2 medium containing kanamycin 50 mg/l, after 4 weeks of incubation. Out of these four, only one callus line survived and proliferated on further incubation on MS2 medium containing kanamycin 50 mg/l (Table 6.4) (Fig. 6.4 A-B). This callus showed intense blue colour when tested for GUS assay (Fig. 6.5 A-B).

Fig. 6.4

A-B. GUS positive callus (derived from bombarded plumular axis) maintained on medium MS + B5 vitamins + TDZ (0.1 mg/l) + Kan (50 mg/l) + glucose 3%.

Fig 6 4

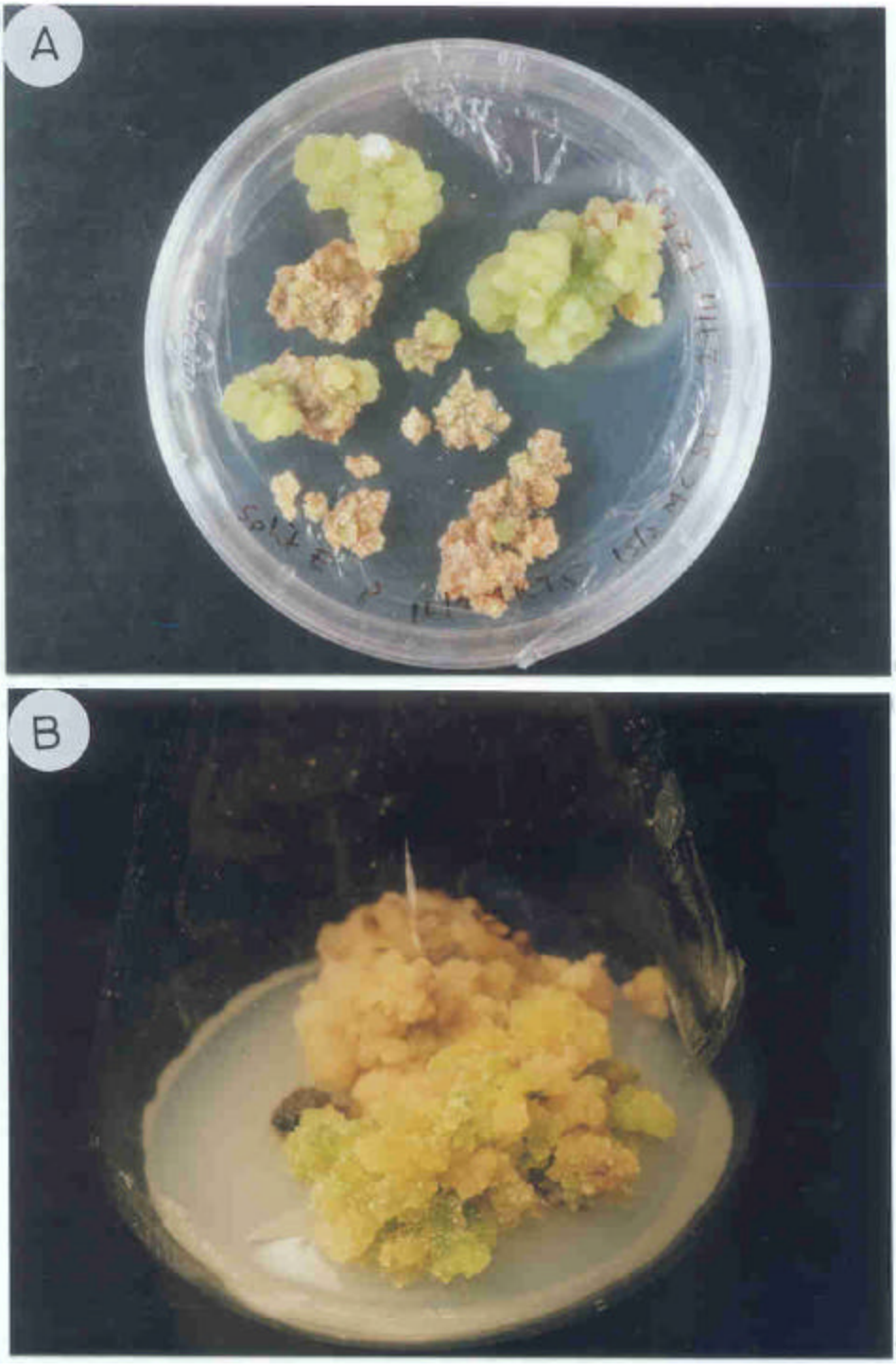


Fig. 6.5
A-B. Gus positive callus (intense blue colour)

Fig. 6.5

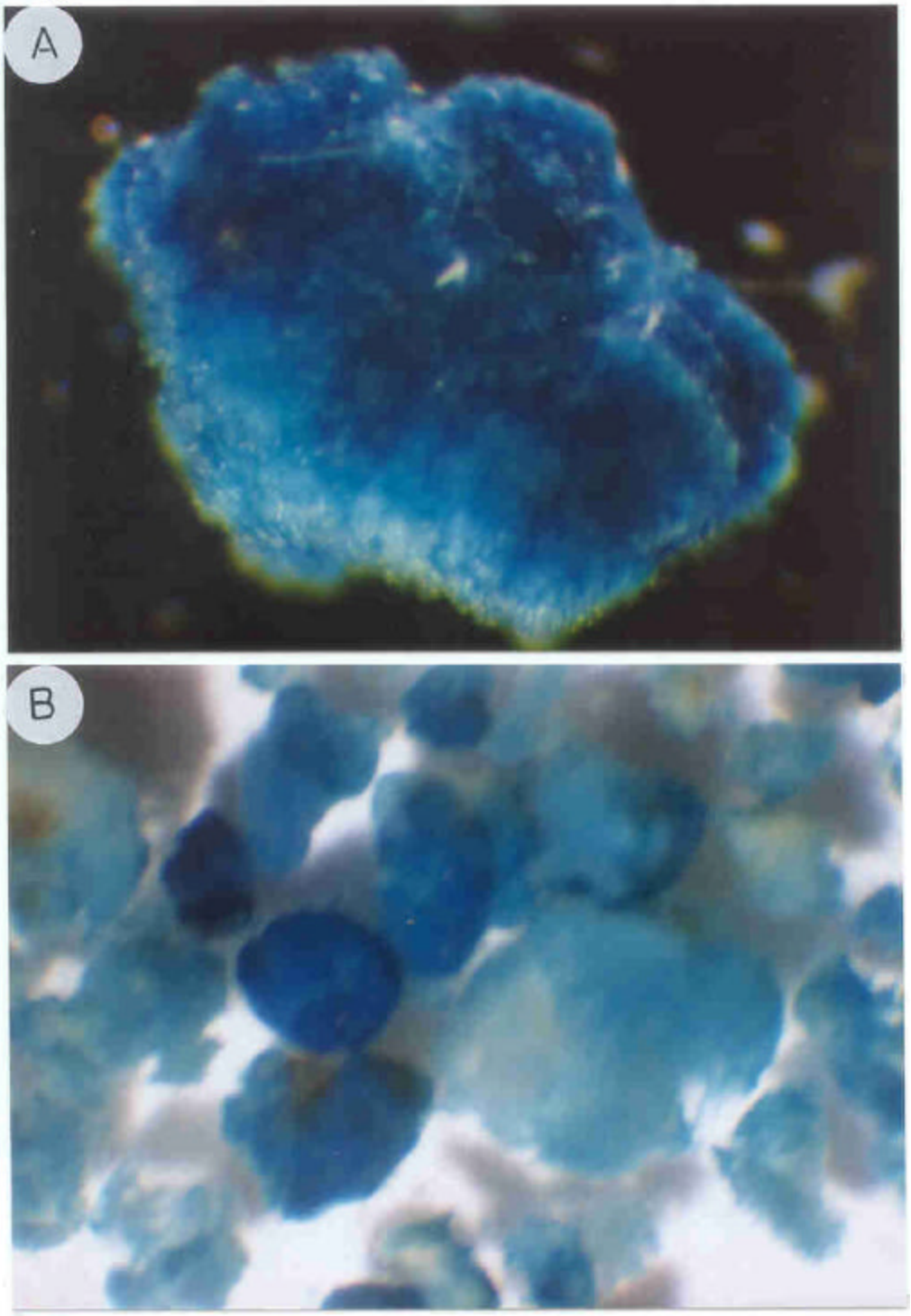


Table 6.4: Development of callus in the plumular axis explants after bombardment.

Cultivar – NHH-44

Explant – plumular axis (2 mm)

Plasmid – p35 GUS INT

Microcarrier – Gold

Rupture disc – 1100psi / 1300 psi

Culture medium – MS basal medium + B5 vit + TDZ 0.1 mg/l + NAA 0.02 mg/l

Number of explants bombarded	Rupture disc (psi)	Number of explants callused after 4 weeks	Number of callus lines survived on kanamycin up to 12 wks
430	1300	-	-
600	1100	4	1

6.3.8. Effect of various phytohormones on morphogenetic response of GUS positive callus

Among the various phytohormones tested to induce morphogenetic response, friable, green callus was observed in MS basal medium containing TDZ (0.1 – 1.0) (Table 6.5). In the medium containing 2,4–D (0.1 – 1.0 mg/l) , 2,4,5–T (0.1 – 1.0 mg/l) NAA (0.01 – 0.1 mg/l) picloram (0.01 – 0.1 mg/l) white loose callus formation was observed whereas in other concentrations of these phytohormone the callus turned brown/black and did not proliferate. The profuse callus growth was obtained on the medium containing TDZ (0.1 mg/l) on transfer to fresh medium every 4 weeks. The callus proliferated into large clumps (Fig. 6.6 A-B). There are several reports on induction of somatic embryogenesis by incorporation of TDZ in culture medium for *Geranium* (Qureshi & Saxena 1992), groundnut (Saxena *et al.* 1992).

Fig. 6.6

A-B. Gus positive callus line proliferating as clumps in suspension cultures (medium MS + B5 vitamins + TDZ (0.1 mg/l) + Kan (50 mg/l) + glucose 3%).

Fig. 6.6

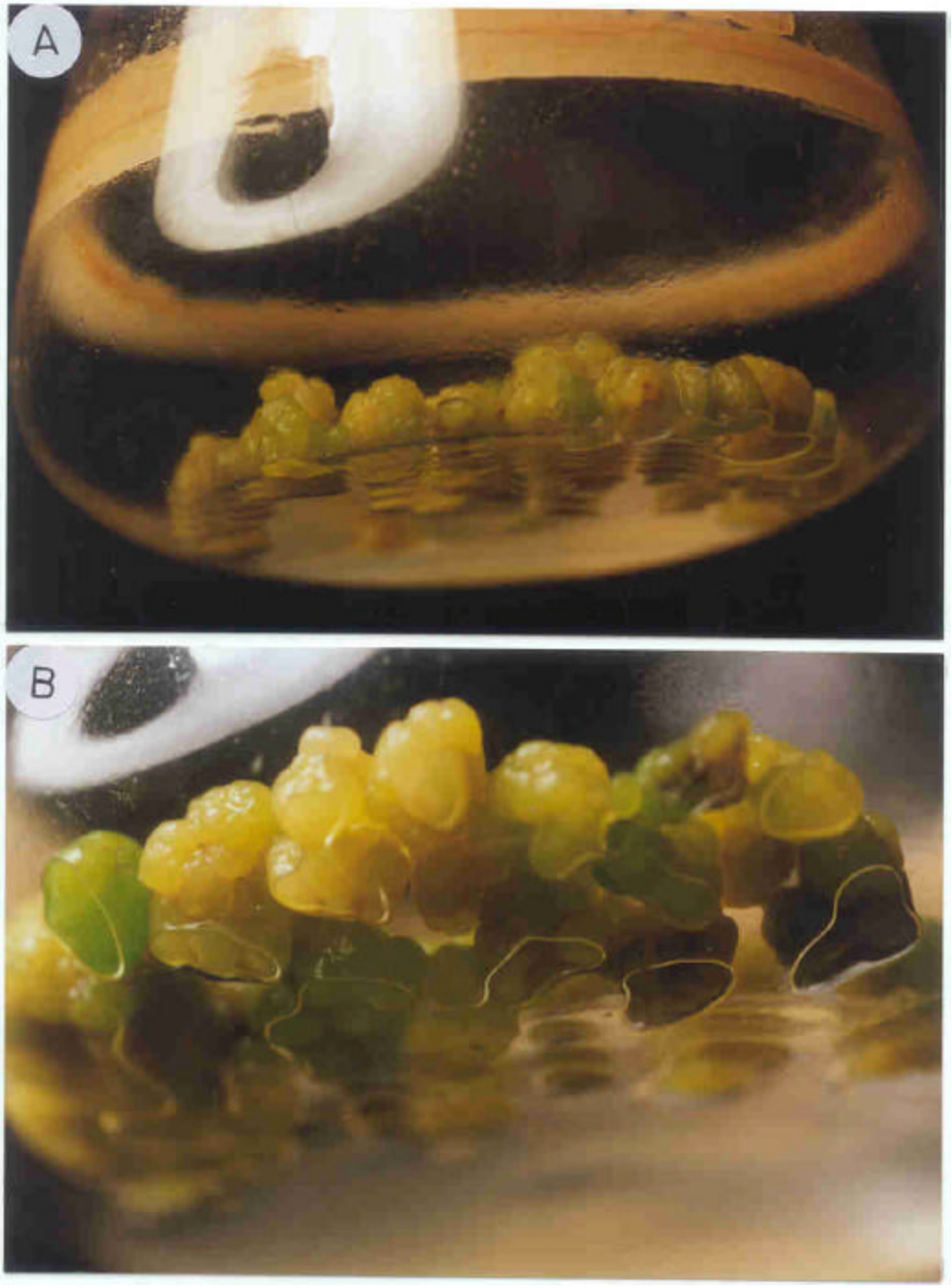


Table 6.5: Effect of various phytohormones on morphogenetic response of GUS positive callus.

S.No	Phytohormones (mg/l)	Response
1.	2,4-D(0.1)	White loose callus
2.	2,4-D(0.5)	White callus
3.	2,4-D(1.0)	White callus
4.	2,4-D(2.0)	Brown callus
5.	2,4-D(5.0)	Brown callus
6.	2,4-D(10.0)	Black callus
7.	2,4,5-T(0.1)	White loose callus
8.	2,4,5-T(0.5)	White callus
9.	2,4,5-T(1.0)	White callus
10.	2,4,5-T(2.0)	Brown callus
11.	2,4,5-T(5.0)	Brown callus
12.	2,4,5-T(10.0)	Black callus
13.	NAA (0.01)	White loose callus
14.	NAA (0.1)	White loose callus
15.	NAA (0.5)	Brown callus
16.	NAA (1.0)	Brown callus
17.	NAA (5.0)	Black callus
18.	NAA (10.0)	Black callus
19.	Pic (0.01)	White loose callus
20.	Pic (0.1)	White loose callus
21.	Pic (0.5)	Brown callus
22.	Pic (1.0)	Black callus
23.	Pic (5.0)	Black callus
24.	Pic (10.0)	Black callus
25.	TDZ (0.01)	Green loose callus
26.	TDZ (0.1)	Friable green callus
27.	TDZ (0.5)	Friable green callus
28.	TDZ (1.0)	Friable green callus
29.	TDZ (5.0)	Hard green callus
30.	TDZ (10.0)	Hard green callus

Structures of various shapes (globular, rod and torpedo) and sizes resembling early stages of somatic embryos were observed after 6 months in the suspension culture medium containing TDZ (0.1 mg/l) (Fig. 6.7 A-C) . All our efforts to develop these structures into later stages of somatic embryos were not successful. These structures either reverted to callus or turned brown in semi-solid and/or liquid media containing different phytohormones (Table 6.6; 6.7).

Embryo maturation and germination are the most difficult steps in cotton regeneration (Gawel and Robacker 1995). Somatic embryogenesis has been achieved in cotton in semi-solid and/or in liquid medium. (Chapter 1 Table 1.10). Gawal and Robacker (1990) could achieve higher number of embryos from embryogenic callus (obtained from petiole in cotton) when cultured as liquid suspension cultures than on semi-solid medium. Finer (1988) has also obtained a high rate of embryo proliferation with suspension cultures of cotton. The slurry of cells formed in suspension cultures has a higher level of accessibility to nutrients and are exposed to more gradual pH changes than those cultured on a semi-solid medium. Also the toxic metabolites that may have been produced by the proliferating cells gets diluted in the suspension cultures (Stevenson *et al.* 1982; George and Sherrinton 1984) and endogenous embryogenesis inhibiting hormones may be leached into the medium more quickly (Gawal & Robacker 1995).

Structures resembling the initial stages of somatic embryos when tested for GUS assay showed intense blue colour (Fig. 6.7 D). Bacterial β-glucoronidase gene (uidA, gusA) commonly referred to as GUS gene, has become the major reporter gene and 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). It is readily detectable at low concentrations. The final cleavage product is insoluble in water; the 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.

Fig. 6.7

A-C. Structures resembling early stages of somatic embryos

D. GUS expression in the somatic embryo like structures (intense blue colour).

Fig. 6.7

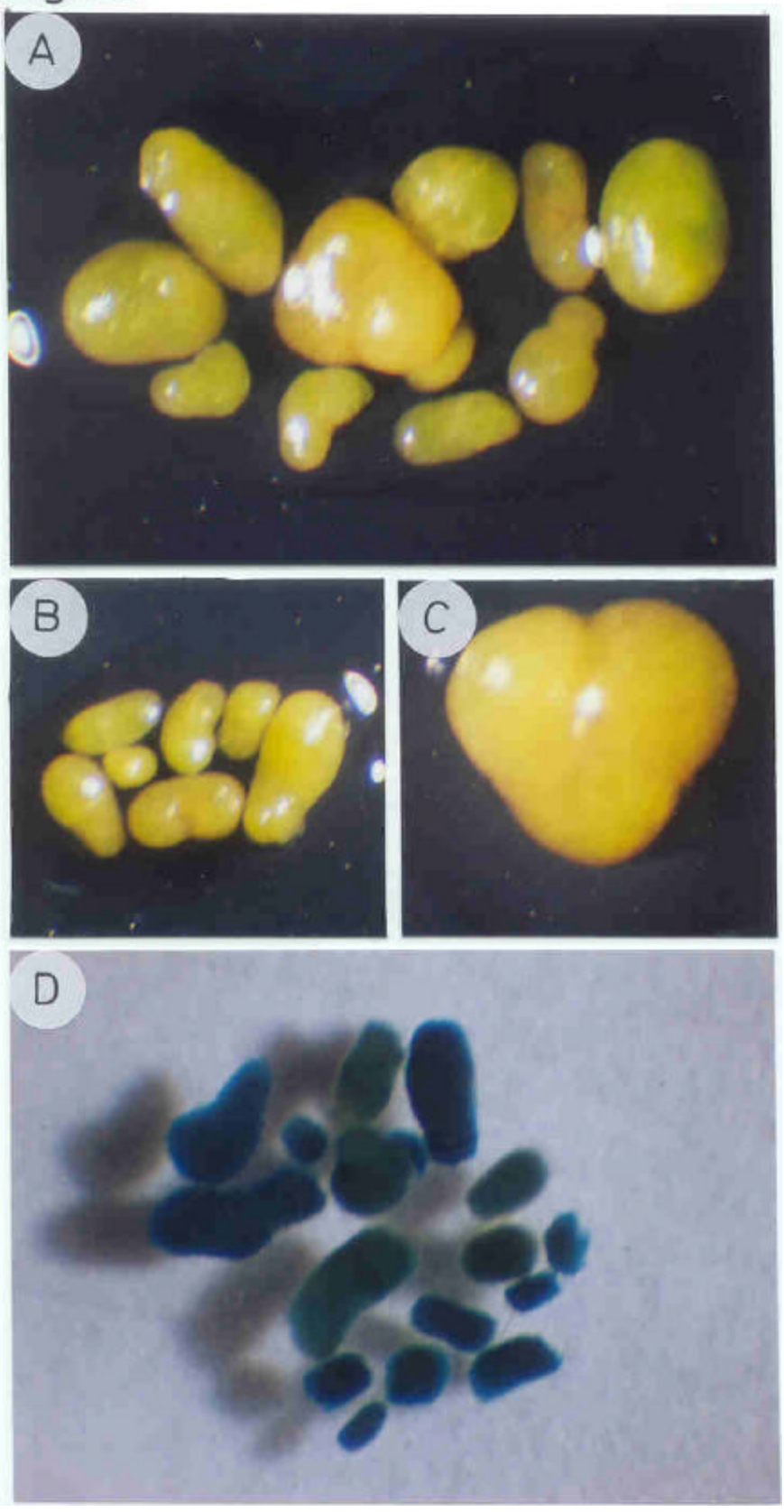


Table 6.6: Effect of different supplements in liquid media tested for the development of somatic embryos from embryo like structures.

No.	Media + supplements in liquid cultures	Response
1	MS + B_5 (Vit)	Callus
2	MS + B_5 (Vit) + Glu (15 mM)	Brown callus
3	MS + B_5 (Vit) + KnO3 (1.9 g/l) + Glu (15 mM)	Brown callus
4	1/2 MS + B_5 (Vit) + Glu (15 mM)	Greenish callus
5	1/4 MS + B_5 (Vit) + Glu (15 mM)	Greenish callus
6	1/4 MS + B_5 (Vit) + Glu (15 mM) + KnO_3	Greenish callus
7	1/4 MS + B_5 (Vit) + GA_3 (0.1 mg/l)	Greenish callus
8	1/4 MS + B_5 (Vit) + GA_3 (0.2 mg/l)	Greenish callus
9	1/4 MS + B_5 (Vit) + GA_3 (0.5 mg/l)	Greenish callus
10	MS + B_5 (Vit) + GA_3 (0.1 mg/l)	Greenish callus
11	MS + B_5 (Vit) + GA_3 (0.2 mg/l)	Brown callus
12	MS + B_5 (Vit) + GA_3 (0.5 mg/l)	Brown callus
13	MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.1 mg/l)	Brown callus
14	MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.2 mg/l)	Brown callus
15	MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.5 mg/l)	Greenish callus
16	1/2 MS + B_5 (Vit) + GA_3 (0.1 mg/l)	Greenish callus
17	1/2 MS + B_5 (Vit) + GA_3 (0.2 mg/l)	Greenish callus
18	1/2 MS + B_5 (Vit) + GA_3 (0.5 mg/l)	Greenish callus
19	1/2 MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.1 mg/l)	Brown callus
20	1/2 MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.2 mg/l)	Greenish callus
21	1/2 MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.5 mg/l)	Greenish callus
22	1/4 MS + B_5 (Vit) + GA_3 (0.1 mg/l) + KnO_3 1.9g/l	Brown callus
23	1/4 MS + B_5 (Vit) + GA_3 (0.2 mg/l) + KnO_3 1.9g/l	Brown callus

24	1/4 MS + B_5 (Vit) + GA_3 (0.5 mg/l) + KnO_3 1.9g/l	Greenish callus
25	1/4 MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.1 mg/l)	Brown callus
26	1/4 MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.2 mg/l)	Brown callus
27	1/4 MS + B_5 (Vit) + Glu (15 mM) + GA_3 (0.5 mg/l)	Greenish callus
28	MS + B_5 (Vit) + Glu (15 mM) + KnO_3 1.9g/l + GA_3 (0.1 mg/l)	Brown callus
29	MS + B_5 (Vit) + Glu (15 mM) + KnO_3 1.9g/l + GA_3 (0.2 mg/l)	Brown callus
30	MS + B_5 (Vit) + Glu (15 mM) + KnO_3 1.9g/l + GA_3 (0.5 mg/l)	Brown callus
31	MS + B_5 (Vit) + $AgNO_3$ (5 µM)	Callus
32	MS + B_5 (Vit) + $AgNO_3$ (10 µM)	Callus
33	MS + B_5 (Vit) + $AgNO_3$ (15 µM)	Brown callus
34	MS + B_5 (Vit) + $AgNO_3$ (20 µM)	Brown callus
35	MS + B_5 (Vit) + ABA (0.1 mg/l)	Turned pale
36	MS + B_5 (Vit) + ABA (0.2 mg/l)	Turned pale
37	MS + B_5 (Vit) + ABA (0.5 mg/l)	Turned pale

Table 6.7: Effect of different supplements in semi-solid media tested for the development of somatic embryos from embryo like structures.

S No.	Media + supplements (semi-solid cultures)	Response
1	MS + B_5 (Vit) + GA_3 (0.1 mg/l)	Callus
2	MS + B_5 (Vit) + GA_3 (0.2 mg/l)	Callus
3	MS + B_5 (Vit) + GA_3 (0.5 mg/l)	Callus
4	MS + B_5 (Vit) + Glu (15 μM) + GA_3 (0.1 mg/l)	Callus
5	MS + B_5 (Vit) + Glu (15 μM) + GA_3 (0.2 mg/l)	Callus
6	MS + B_5 (Vit) + Glu (15 μM) + GA_3 (0.5 mg/l)	Callus
7	MS + B_5 (Vit) + KNO_3 (1.9g/l) + GA_3 (0.1 mg/l)	Callus
8	MS + B_5 (Vit) + KNO_3 (1.9g/l) + GA_3 (0.2 mg/l)	Callus
9	MS + B_5 (Vit) + KNO_3 + GA_3 (0.5 mg/l)	Callus
10	MS + B_5 (Vit) + Glu (15 μM) + KNO_3 (1.9 g/l)	Callus
11	MS + B_5 (Vit) + Glu (15 μM) + KNO_3 (1.9 g/l)	Callus

6.4. Conclusion

Transient expression of β-glucoronidase gene by particle bombardment in plumular axis of cotton cultivar NHH-44 has been achieved with plasmid p35SGUSINT. The GUS expression was achieved in large number of explants when bombarded with 1100 psi rupture disk at 6 cm target distance.

Leaf bits taken from the shoots survived on selection pressure for 12 weeks were found to be GUS negative. GUS positive callus was obtained from the bombarded explants on MS salts + B_5 vitamins + TDZ 0.1 mg/l + NAA 0.01mg/l + 3 % glucose in 12 weeks. The callus proliferated as clumps and development of structures resembling early stages of somatic embryos was achieved in liquid medium supplemented with TDZ 0.1 mg/l. These structures failed to develop into further stages of somatic embryos and plantlets.

A part of this chapter entitled "Transient expression of β-glucuronidase in embryo axes of cotton (*Gossypium. hirsutum* L.) by *Agrobacterium* and particle bombardment methods" by Banerjee A. K., Agrawal D. C., **Nalawade S. M.**, and K. V. Krishnamurthy has been communicated for publication to Biologia Plantarum

Summary

Summary

Cotton is one of the most important multipurpose crops valued for its lint (fiber), oil, seed meal, hulls and several other by-products. The cultivated and wild varieties of cotton belong to genus *Gossypium* (family Malvaceae). Cotton is grown in 90 countries on 32.6 million hectares 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). India is one among the top five cotton producing countries in the world.

To meet the increasing demands of superior quality cotton by textile industry and also to restrict the damage caused to environment by excessive use of pesticides, there has been an increased interest in quantitative and qualitative improvement of cotton varieties. Conventional breeding techniques have limitations to meet these objectives, often due to incompatibility barriers between cotton species. The application of biotechnological techniques in breeding programs provide alternative avenues for cotton improvement through direct manipulation of genes and yield improvements not possible to achieve through classical breeding techniques. However, an efficient plant regeneration protocol is essential pre-requisite for development of transgenic plants. The majority of the reports published mainly from the USA are on Coker varieties that are not cultivated in India. There were no reports on *in vitro* plant regeneration from juvenile explants of Indian cutlivars of cotton when the study was initiated. Therefore, the present study was undertaken to develop an efficient plant regeneration protocol using various explants derived from seeds, *in vitro* raised seedlings and field-grown mature plants. Yet another objective of the work was to study application of explants with pre-existing meristems for genetic transformation in cotton by particle bombardment method.

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

A: *In vitro* propagation and plant regeneration from embryo axis explants

1. A simple and rapid plant regeneration protocol from intact embryo axis and split embryo axis explants of six Indian cultivars of cotton has been developed.

2. Incorporation of charcoal (0.25 %) in the culture medium and incubation of cultures at higher temperatures (30 ± 2 °C) resulted in higher number of explant forming shoots.
3. High frequency plant regeneration (68 – 82 %) from the axenic nodal segments has been achieved in the MS basal medium devoid of phytohormones in six cultivars.
4. Induction of multiple shoots from the plumular axis explants of three cultivars viz. DCH-32, LRA-5166 and DHY-286 was achieved on a medium containing MS salts + B5 vitamins + BA 0.1 mg/l + NAA 0.02 mg/l. Further shoot proliferation was achieved on transfer of multiple shoot bunches to the fresh medium and on further incubation for 10 weeks.
5. Rooted shoots from all the experiments survived after hardening in greenhouse and grew to maturity after transfer to field.

[*Parts of this work has been communicated as two papers (**Plant Cell Tissue and Organ Culture** and **Biologia Plantarum)** and also an Indian patent has been filed - See author's publication section*].

B. Plant regeneration from explants consisting of pre-existing meristems from *in vitro* grown seedlings

1. A protocol for clonal propagation using explants with pre-existing meristems derived from *in vitro* grown seedlings of five Indian cotton cultivars has been developed.
2. Induction of multiple shoots from five explants derived from *in vitro* grown seedlings has been achieved. The sequence of two media MS1 (MS salts and vitamins (MS basal medium) + BA 1.0 mg/l + NAA 0.2 mg/l + sucrose 2%) for the initial culture of 3 weeks followed by MS2 (MS basal medium + BA 1.0 mg/l + glucose 3%) for the second and third passages of 3 weeks each were found to be best media for induction of multiple shoots.
3. The maximum number of shoots (6.38) per responding explant and the maximum percentage of response (90%) was achieved with 21-d old seedlings.
4. The morphogenetic response varied greatly among the different explants and cultivars tested. The best shoot multiplication rate was observed with split cotyledonary node and petiole base explants in all the five cultivars.

5. The larger culture vessel with more medium (250 ml capacity conical flasks with 50 ml of medium) favored more number of multiple shoots compared to test tubes (25 mm x 150 mm with 20 ml medium) in all the five cultivars.
6. The maximum number of shoots (19.7) per responding explants was observed in split cotyledonary node (SCN) explants of cultivar LRK-516 when cultured in flasks.
7. Rooting of i*n vitro* shoots was achieved on semi-solid MS basal medium devoid of phytohormones. All tissue culture plantlets survived after hardening under greenhouse conditions.

[*Part of this work has been published* ***(In Vitro Cellular and Developmental Biology-Plant)*** *and also has been applied for an Indian patent - See author's publication section*].

C. Clonal propagation using nodal segments from field-grown mature plants

1. A protocol has been developed for plant regeneration from nodal segments obtained from mature field-grown cotton plants of cultivars DCH-32 and NHH-44.
2. The maximum percentage (82.5 %) of sprouting in explants of cultivar DCH-32 was recorded in MS basal medium devoid of phytohormones.
3. The maximum number of multiple shoots from nodal segments devoid of sprouted shoot (mother explants) of cultivar DCH-32 (5.1 shoots per responding explants) and NHH-44 (4.3 shoots per responding explants) was achieved in MS basal medium supplemented with BA 0.5 mg/l. The multiple shoots elongated on the same medium.
4. Larger culture vessel (250 ml capacity conical flask) had a positive influence on induction of more number of multiple shoots in the mother explants of both the cultivars DCH-32 and NHH-44 DCH-32 compared to test tubes.
5. *In vitro* shoots could be rooted on MS basal medium devoid of phytohormones. The hardened plants transferred to field showed normal plant growth and boll formation.

[*Part of this work has been accepted for publication* ***(In Vitro Cellular and Developmental Biology-Plant)*** *- See author's publication section*].

D. Application of embryo axis explants in transformation of cotton via particle bombardment approach

This study was carried out to optimize different parameters of particle bombardment method for transient gene expression in plumular axis explants of cotton cultivar NHH-44.

1. Transient gene expression of β-glucoronidase (GUS) in plumular axis explants of cotton cultivar NHH-44 was achieved with plasmid p35SGUSINT.
2. Structures resembling early stages of somatic embryo were obtained in suspension cultures on MS + B_5 vitamins + TDZ 0.1 mg/l.
3. Intense GUS activity was observed in callus tissue and in structures obtained in suspension cultures
4. The maximum number of explants (27.1 %) with GUS expression was achieved in cultivar NHH-44 with gold microcarriers, 1100 psi rupture disk at 6 cm target distance .

[*Part of this work has been communicated for publication to* ***Biologia Plantarum*** *- See author's publication section*].

In nutshell, efficient and reproducible plant regeneration protocols have been developed with explants having pre-existing meristems from seeds, *in vitro* raised seedlings and field-grown mature plants. Transient expression of GUS gene in plumular axis explants of cultivar NHH-44 has also been achieved.

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