GENETIC TRANSFORMATION IN WHITE JUTE
(*Corchorus capsularis*)

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CERTIFICATE

*This is to certify that thesis entitled, "**Genetic Transformation in White Jute (Corchorus capsularis)**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in GENETICS AND PLANT BREEDING,** embodies the result of a piece of bona fide research work carried out by **SHAIKH TAZUL ISLAM,** Registration No.25281/00618 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: June, 2007
Place: Dhaka, Bangladesh

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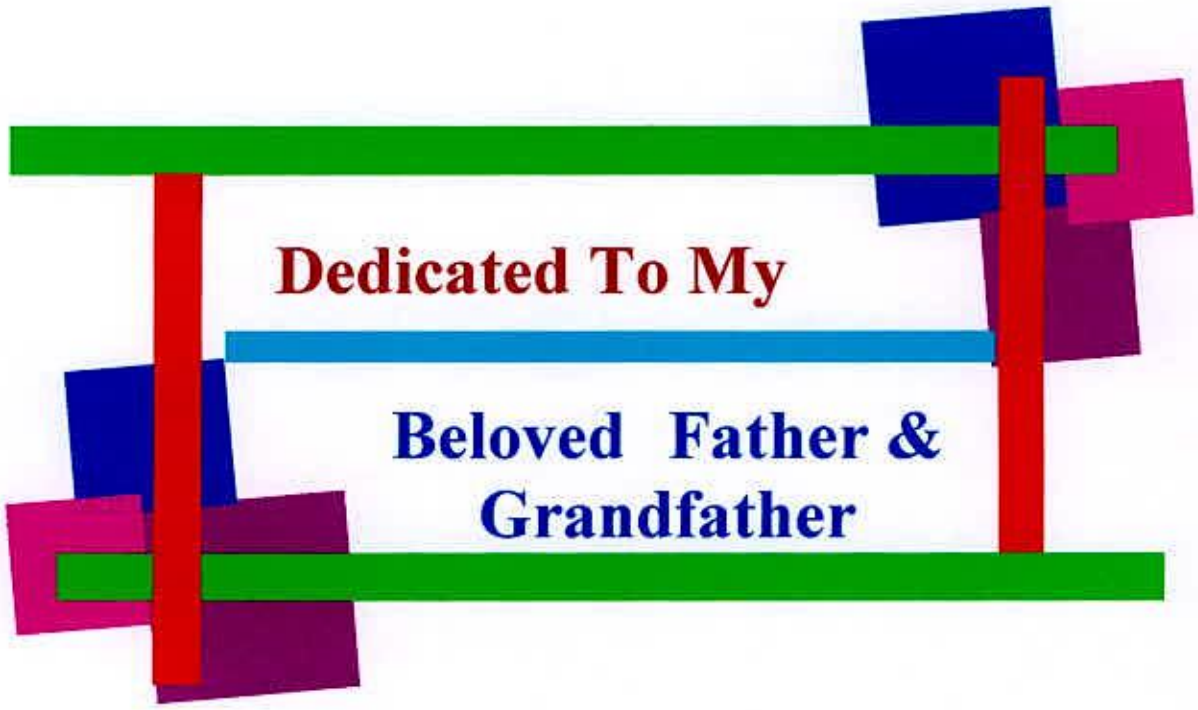
Dedicated To My
Beloved Father &
Grandfather

LIST OF ABBREVIATIONS

Abbreviation		Full word
%	:	Percentage
0.1 N	:	0.1 Normal
BAP	:	6-Benzyl amino purine
BBS	:	Bangladesh Bureau of Statistics
CaMV	:	Cauliflower Mosaic Virus
CIP	:	International Potato Centre
DMRT	:	Duncan's Multiple Range Test
DMSO	:	Dimethyl sulfoxide
dw	:	Distilled water
et al.	:	et alu = other people
eg	:	Exempli gratia (Latin; for example)
etc	:	Et cetra (means and the rest)
FAO	:	Food and Agriculture Organization
Fig.	:	Figure
g	:	Gram
gl^{-1}	:	Gram per litre
GUS	:	B-glucuronidase
HCl	:	Hydrocloric acid
$HgCl_2$	:	Mercuric cloride
h	:	Hour (s)
i.e.	:	ed est (means That is)
IAA	:	Indol-3-acetic acid
IARI	:	Indian Agricultural Research Institute
IBA	:	Indol-3-butaric acid
ICRISAT	:	International Crop Research Institute for the Semi-arid Tropics
IRRI	:	International Rice Research Institute
j.	:	Journal
l	:	Litre
Lux	:	Unit of illumination
LB	:	Luria Broth
M	:	Molar or Manitol
min	:	Minute (s)
mg	:	Milligram
mg/l	:	Milligram per litre
ml	:	Millititre
ml/l	:	Millilitre per litre

Abbreviation		Full word
MS	:	Murashige and Skoog (1962)
Na_2-EDTA	:	Sodium salt of ferric ethyleve diamine tetraacetate
NAA	:	α-Napthelene acetic acid
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
No.	:	Number
NS	:	Non-significant
pH	:	Negative logarithm of hydrogen ion concentration (-log [H^+])
req	:	Required
T-DNA	:	Transfer DNA
UK	:	United Kingdom
USDA	:	United States Department of Agriculture
UV	:	Ultra violet
var.	:	Variety
via	:	By way of
vir	:	Virulence region
Viz.	:	Namely
X-gluc	:	5-bromo-4-choloro-3-indolyl-β-D-glucuronide
YMB	:	Yeast extract Mannitol Broth
µg	:	Microgram
Vol.	:	Volume
kg	:	Kilogram
µl	:	Microlitre

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SAU, Dhaka *The Author*

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GENETIC TRANSFORMATION IN WHITE JUTE
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BY

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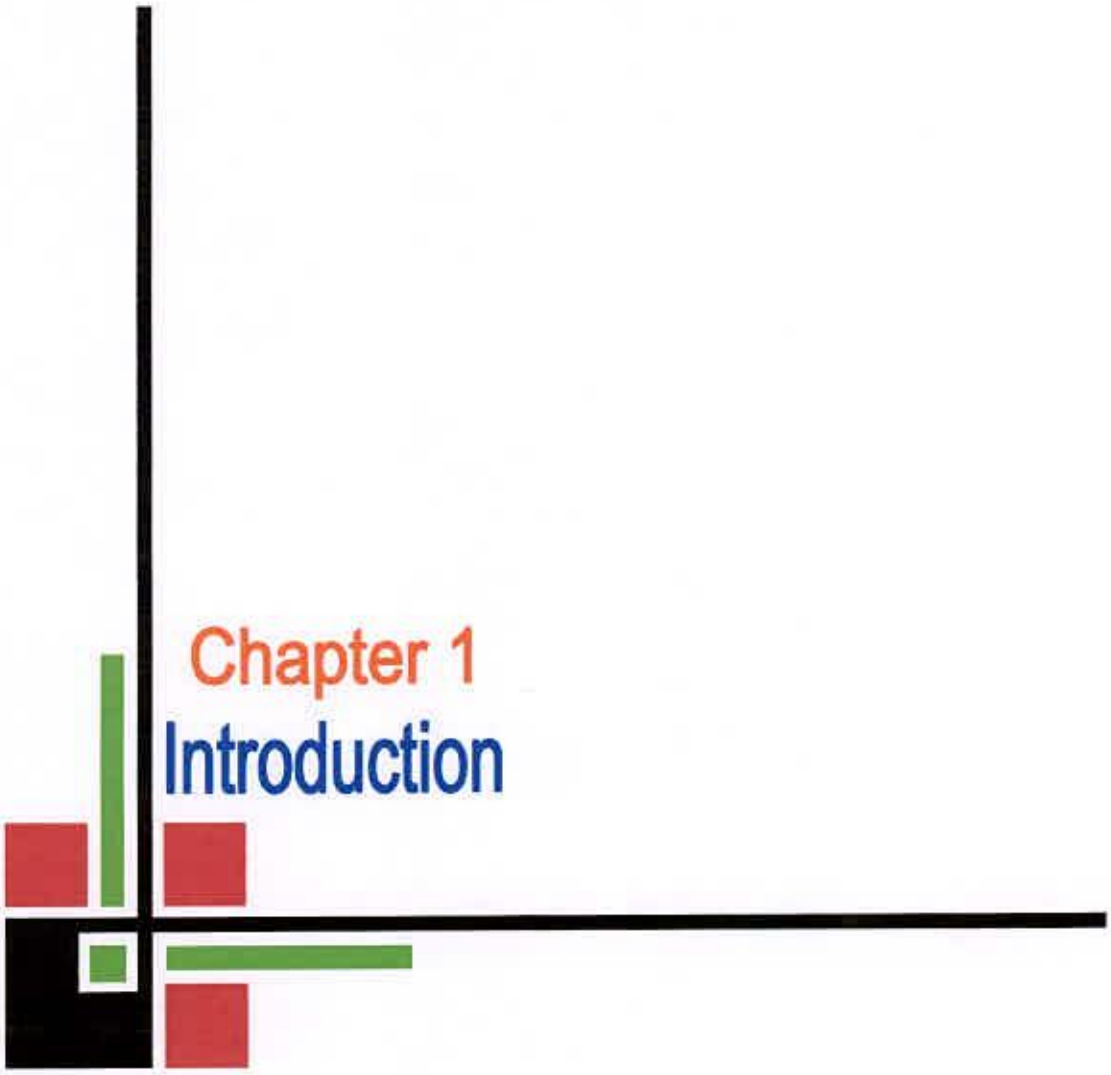
ABSTRACT

Two section of experiment were conducted during the period from July 2006 to June 2007 in the Genetic Engineering Laboratory of Genetic Resources and Seed Division, Bangladesh Jute Research Institute (BJRI), Dhaka. In this section-A, *C. capsularis* was used to investigate their *in vitro* regeneration potentiality and in the section-B, two varieties of *C. capsularis* were used to observe their transformation ability. Cotyledons (with attached petioles) were used as explants. One of the major constraints of getting plant regeneration from the explants of *C. capsularis* was the production of healthy seedlings *in vitro*. Seeds of *C. capsularis* germinated on both agar supported hormone free MS medium and cotton supported hormone free liquid MS medium. The percentage of seeds germinated on cotton-supported medium was found to be much higher than seeds germinated on agar-supported medium. In the section-A, seed germination percentage was found to be the highest in CVE-3 in cotton-supported medium (88.89%) than agar-supported medium (77.77%). Among the phytohormone combination, MS+ 2 mg/l BAP + 0.5 mg/l IAA showed the highest shoot regeneration (83.33%). Plant regeneration was also observed in the presence of different percentages of surfactant (Pluronic F-68) and different concentration of $FeSO_4$ and it was found that 0.08% surfactant and 28 mg/l $FeSO_4$ gave highest result respectively. In the section-B, an efficient and reproducible protocol for the production of transgenic jute plant was developed by inoculating cotyledonary petioles with *Agrobacterium tumefaciens* strain LBA4404 carrying a binary vector pBI121, which contains selectable marker gene *nptII* conferring resistance to kanamycin and the GUS reporter gene. After co-cultivation and selection, histochemical GUS assay was performed in two varieties (*viz.* CVE3 and CVL1). In the transformed explants, GUS reporter gene was expressed showing blue colour in the explant tissues. Non-transformed explants did not show any colour. Among the varieties, CVE-3 showed the highest response to GUS assay (90%).



Chapter 1
Introduction

INTRODUCTION

Jute belongs to the family of Tiliaceae. There are two cultivated species, one is *Corchorus capsularis* L. and another is *Corchorus olitorius* L. It produces a bast (bark) fiber, which is one of the most important vegetable fibers next to cotton (Singh, 1976). Commonly *Corchorus capsularis* L. is known as white jute.

Jute is one of the most important cash crops of Bangladesh. It occupies 5th position after rice, pulses, oil seeds and wheat in respect of cultivated area (BBS, 2003). Bangladesh is not only the second largest producer of jute but she produces the best quality jute and leads the export market. In the year of 2001-2002, the acreage, production and yield of jute were 1128 thousand acre, 859 thousand bales and 762 kg/acre respectively (BBS, 2003). It is extensively used in the manufacture of different types of packing materials for various agricultural and industrial products. Jute not only constitutes a major currency earner, but is also a major source of employment, which is of prime importance to the rural economics of Bangladesh.

Commercially, jute is often referred to as the "Golden fiber of Bangladesh", because of its immense contribution for the economy of this country. Considerable size of the total population of our country is engaged directly and indirectly in production and processing of jute. Jute exports constitute a major source of foreign exchange (12-13%) earning in Bangladesh. During the year of 2001-2002, Bangladesh exports 619000 tons of raw jute and jute goods and earned about Tk. 16908.00 million (BBS, 2003).

Cultivation of jute in Bangladesh is increasingly shifting to less productive land with marginal care. Thus creating challenges in dealing with new emerging production constraints. In every year, about 7 lac 67 thousand bales of jute are damaged by insect-pest (Ahmed *et al*, 1993). Diseases have also an adverse effect on yield (about 5 lakh bales damaged by diseases). Abiotic stresses like drought, flood, low temperature etc. are detrimental to this crop. With the launching of global campaign for environmental awareness internatior al opinion is being created on jute for its expanded production and use, as it is biodegradable and friendly to the environment. Jute is a plant, all parts of which have extensive uses. To maintain a sustainable improvement in jute productivity under less favorable environment can only be achieved with a constant flow of new genetic materials. The existing variability for constraints, like insect-pest and diseases, poor soil fertility, water stress, fiber quality and photo-intensity etc. is a serious issue that needs to be addressed (Aggarwal, 2000). One of the major constraints to increase jute productivity is the non-availability of modern varietics with improved plant types.

At present, very less success can be achieved for jute production through the conventional breeding methods. New genotypes need to introduce in the field of jute breeding. Although a number of high yielding variety of jute have been released from the Bangladesh Jute Research Institute (BJRI) through conventional breeding techniques, these techniques still have many limitations. It is therefore, very important to explore other means of modern scier tific techniques for example, tissue culture or genetic engineering to accelerate the pace of varietals improvement.

The chances for availability of new genotype of jute with disease resistance in nature are very remote unless new techniques are launched to create variability. Biotechnology is a recently developed novel approach and therefore it is very important to explore these techniques for varieties improvement of jute.

The pre-requisite of genetic transformation in jute was to establish an efficient system from explants to matured fertile plants. Plant regeneration from the cotyledonary petioles was reported earlier from *C. capsularis* (Khatun *et al.* 1992) and from the cotyledonary petioles of *C. olitorius* (Khatun, *et al.*, 2003). Transformation of higher plants has been accomplished by different methods (Gardner 1993, Gasser and Fraley 1989, Paszkowski *et al.* 1989). The most common and efficient one utilizes non-ontogeny *Agrobacterium* strain as a gene vector (Lindsey, 1992).

White jute (*Corchorus capsularis*) is susceptible to root-knot nematodes and spiral borers. This species is also susceptible to stem rot and leaf mosaic diseases. Genes are available against stem borer, fungus and viral diseases, which could be inserted in jute in future through genetic transformation. Before developing a protocol for gene transfer in jute, there is a need to develop an efficient and repeatable plant regeneration system from jute explants. The developed protocol for plant regeneration from jute explants then would be used for insertion of agronomically important genes in jute plants in future. At present, marker genes e.g. kanamycin and GUS genes would be used for jute transformation.

Genetically engineered foreign genes have been successfully transferred into several agriculturally important crop plants including jute by *Agrobacterium*-mediate transformation (Bajaj, 1989). Recently, plants have been engineered to be resistant to herbicide, viruses and insects. Genetic transformation could be one option for the improvement of jute varieties. A vector system is therefore, needed to be developed for the production of transgenic jute expressing agronomically important traits like jute plants with resistant to insect or fungus, with suitable marker genes. With this view in mind, the present research work has been undertaken. The present study has been divided into two separate sections. This are-

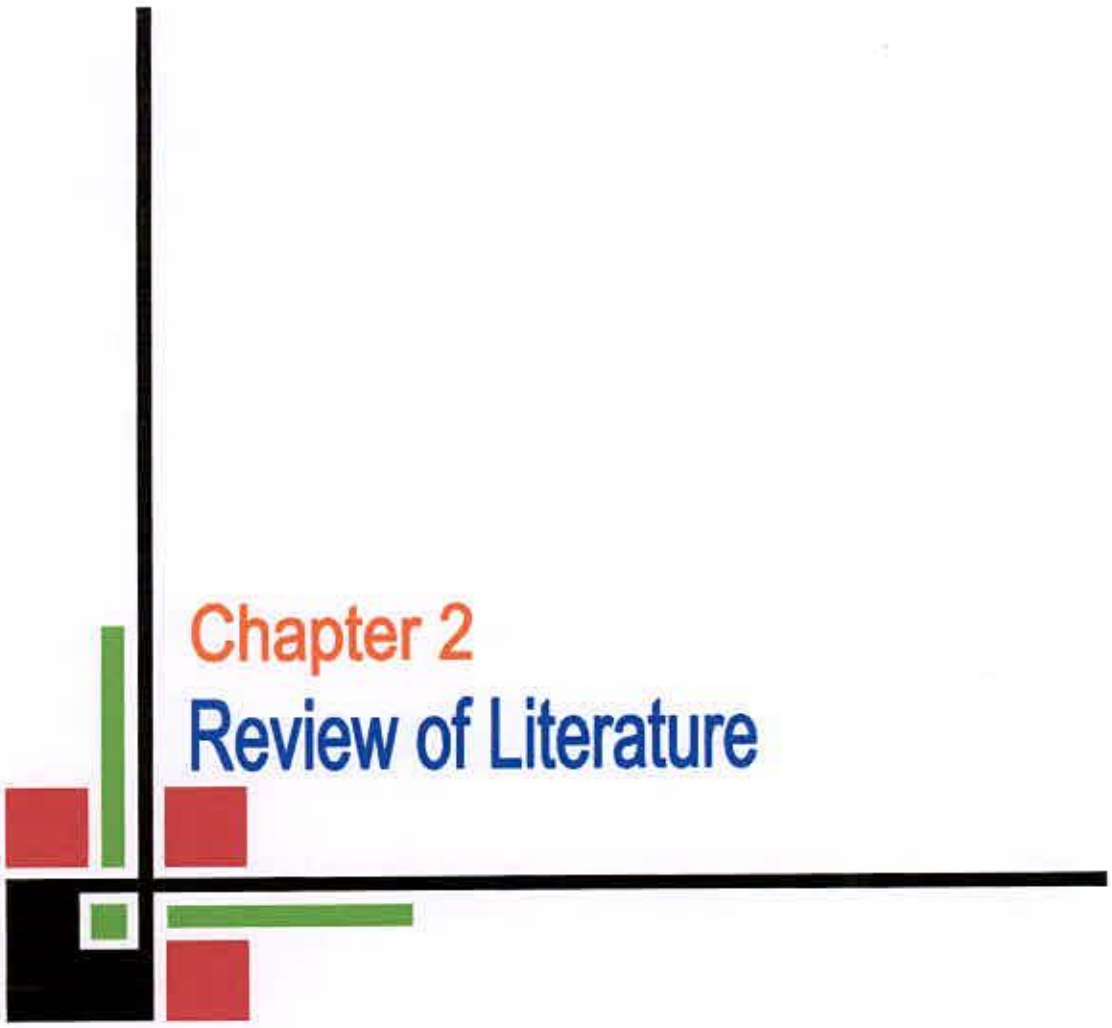
Section-A: *In vitro* regeneration potentiality of *C. capsularis varieties.*

Section-B: *Agrobacterium*-mediated genetic transformation of *C. capsularis*

The objectives of the present research are:

i. To develop a suitable and reproducible protocol for *in vitro* plant regeneration in *C. capsularis.*

ii. To develop a reproducible and efficient protocol for the insertion of foreign genes into *C. capsularis* through *Agobacterium tumefaciens* vectors.



Chapter 2
Review of Literature

Jute is the most important fiber crop of Bangladesh. The crop received much attention by a large number of researchers on various aspects of production and utilization. Improvements of crop plants like jute through conventional method require long time. Plant biotechnology now a day offers many opportunities for breeders with chances to solve certain breeding problems at cellular level. Biotechnological research on jute has been initiated in early sixties (Islam, 1964). However, output is still very limited. Recent advances in tissue culture and recombinant DNA technology have opened new avenues in transformation of higher plants, which consequently produced many transgenic plants with new genetic properties. Establishment of an efficient plant regeneration system from the explants of jute is a prerequisite to create variability and to introduce foreign genes into this crop through genetic transformation (Khatun, 1998). Brief review of work done on plant regeneration and transformation of jute and allied fibers is summarized bellow.

2.1 *In vitro* regeneration potentiality of *C. capsularis* varieties

2.1.1 Concept of tissue culture

Conventional techniques are lengthy processes for crop improvement. The techniques of plant tissue culture have been developed as a new and powerful tool for crop improvement (Carlson, 1975, Razdan and Cocking 1981) and received wide attention of scientists (D'Aamato1978, Skirvin 1978, Larkin and Scowcroft 1982). Regeneration from different explants (leaf, stem, cotyledon and hypocotyls) on defined nutrient media under sterile conditions is the basis of plant tissue culture. When explants of a plant are culture in a defined medium, an

undifferentiated collection of cells arise which then develop into whole plants from this undifferentiated callus is known as regeneration. Tissue culture technique is now used extensively in many national and international organizations, such as CIP, IARI, ICRISAT, USDA, where programs of crop improvement are in progress for development of different crops.

2.1.2 Tissue culture of jute

In vitro plant regeneration has been quite difficult among the species *Corchorus* through tissue culture technique. It appears that jute is a notorious recalcitrant plant and regeneration from it is sporadic. Regeneration has only been reported from meristematic tissue but not from totally differentiated tissue, like callus. Where there are reports of regeneration from cotyledon or hypocotyls derived callus, there are usually portions of meristematic tissue left from where regeneration actually occurs.

Tissue culture research in jute was started in 1964, when Islam (1964) cultured interspecific hybreed embryo (*Corchorus capsularis* × *Corchorus olitorius*) and hybrid plants were obtained. Plant regeneration of jute from meristem (Rahman *et al*., 1985), cotyledons (Rahman *et al.* 1985; Khatun *et al.* 1992; Ali, 1992), leaf (Islam, 1981), plumule (Das *et al.* 1996), hypocotyl (Khatun *et al.* 1992; Ghosh and Chatterjee, 1990; Seraj *et al.* 1992), apical meriatems (Rahman *et al.* 1985) and anther culture [IBFC (CAAS), 1974; Islam *et al.* 1981] have been reported.

2.1.2.1 *In vitro* seed germination

Healthy seedling production was one of the major criteria for plant regeneration. However, very few work and attention has been paid so far on *in vitro* seed germination of jute. Some literatures related to *in vitro* seed germination are cited bellow:

Naher *et al.* (2003) conducted an experiment to study the seed germination percentage of varieties of *C. capsularis* (vars. CVE-3, CVL-1, D-154, CC-45, BJC-83, BJC-7370, BJC-718 and BJC-2142) on hormone free agar-solidified MS basal medium and clinical cotton-based MS liquid medium. They reported that the percentage of seed germination among the varieties was found higher on cotton-based medium than the agar-based medium. Naher *et al.* (2003) also reported that the highest percentage of seed germination was found in the variety CVE-3 (97.33%) on cotton-based medium and the lowest (86.33%) on agar-based medium.

Khatun (2001) conducted an experiment to study the germination percentage of varieties *C. capsularis* (vars. CVL-1, CVE-3 and D-154) on hormone free agar-solidified MS basal media and cotton-based MS liquid medium. She reported that among the varieties, percentage of seed germination was found the highest (98.66%) on cotton-supported medium and the lowest (68.66%) on agar-supported medium in the variety CVE-3.

2. 1. 2 .2 Callus induction

A callus is an amorphous mass of loosely arranged thin walled parenchyma cells arising from the proliferating cells of parent tissue (Dodds and Robert, 1990). Callus induction from different explants of various jute (*C. capsularis*) varieties in the combinations of growth regulators were reported by several workers. The most relevant literatures related to callus induction have been reviewed here:

2.1.2.2.1 Effect of explants

Rahman *et al.* (1985) showed that callus initiated from both apical meristems and cotyledons of var. D-154 of *C. capsularis*, when cultured on BAP and tyrosine fortified MS media forms shoot.

Khatun *et al.* (1992) reported cotyledon-derived callus. They used phytohormones BAP and IAA with MS medium to set multiple shoots from cotyledon-derived calli. Ali (1992) also recorded similar observation. Seraj *et al.* (1992) reported that callus initiated from hypocotyls of D-154 and CVL-1 of *C. capsularis* when cultured on BAP and tyrosine fortified MS medium. They also used on antioxidant NDGA (nordihydroguaiaretic acid).

2.1.2.2.2 Effects of growth regulators

Tewari *et al.* (1999) reported that 2, 4-D induced callus initiation in 100% explants when cotyledons, segments of hypocotyls and roots of white jute (*C. capsularis*) were cultured on MS medium supplemented. Khatun (2001) cultured *in vitro* grown cotyledons (with attached petioles) of *C. capsularis* in agar solidified MS medium supplemented by 0.5 mg/l IAA and different concentration of BAP (2, 3, 4 or 5 mg/l) and noted least performance in callus induction and shoot regeneration in the combination of MS + 0.5 mg/l IAA and 2 mg/l BAP.

2.1.2.2.3 Maintenance of callus

Very little work and attention has been paid so far on maintenance of callus of jute.The organogenic callus of *C. capsularis* (Var. D-154 and CVL-1) when rich in large starch granules, was transferred to MS basal medium, and if differentiated into single or multiple shoots (Seraj *et al.* 1992).

2.1.2.3 Shoot regeneration

The totipotency of somatic cells has been explained in vegetative propagation of plant species. *In vitro* studies have revealed that most plants would differentiate shoots and roots from somatic as well as reproductive tissues. Whole plant regeneration from cultured cells may occur either through shoot-end differentiation of plant from callus has been reported by different workers. The literatures closely related to *in vitro* regeneration of jute are cited below:

Naher *et al.* (2003) reported multiple shoot regeneration from cotyledons with attached petioles cultured on MS medium supplemented by 2 mg/l IAA and found the highest in CVE-3 (91%) and the lowest in BJC-7370 (43.33%). It has been reported that CVE-3 showed higher performance (91% cotyledons were responded) in case of multiple shoot regeneration (32 shoot/explant) among 10 varieties of *C. capsularis*.

Khatun (2001) conducted an experiment on six varieties of jute (CVI-1, CVE-3, D-154, CC-45, and BJC) and observed that the frequency of shoot production varied greatly among the varieties. She reported that CVE-3 (88.33%) showed the best performance in shoot regeneration.

Khatun *et al.* (2003) reported that the cotyledonary explants of *C. olitorius* produced multiple shoot when cultured in MS medium with 0.5 mg IAA/l and 3 mg BAP/l and also reported that the best *in vitro* response for shoot regeneration was obtained from 0-9897 (59.33%), when used four varieties of *C. olitorius* (var. 0-4, 0-9897, OM-1 and O-72).

2.1.2.5 Effect of pH on plant regeneration

Naher and Khatun (2004) reported that two varieties of *C. capsularis* (vars. CVL-1 and D-154) performed differently on shoot regeneration in different pH levels (e.g. 3.5, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) in association with MS plant regeneration medium. They found that D-154 responded for maximum shoot regeneration at pH 5.0 (65.00%) and CVL-1 at pH 7.0 (63.33%). They also reported that shoot regeneration percentage of D-154 gradually decreased as pH levels were increased and shoot regeneration of CVL-1 gradually increased as the pH levels were increased.

2.1.2.6 Influence of surfactants (Pluronic F-68) on plant regeneration from cotyledons of *C. capsularis*

Khatun *et al.* (1992) conducted an experiment of stimulation of differentiation in jute cotyledon cultured with Pluronic F-68. They reported that the addition to MS-based medium of 0.1 or 0.5% (w/v) of either commercial grade Pluronic F-68 or a purified fraction obtained by passage through silica gel, stimulated shoot production from the petioles of cotyledons of *C. capsularis* vars. D154 and C134. This effect was pronounced with C134, because of the failure of control cotyledon to differentiate into shoots in MS medium without Pluronic F-68. The implications of these results are discussed in relation to the potential value of non-ionic surfactants as additives to plant culture media for stimulating growth and differentiation.

Lowe *et al.* (1993) showed that a novel approach to the growth of cultured plant cells, tissues and organs by supplementation of culture media with low concentrations (<1.0% w/v) of surfactants is discussed. Studies using *Arabidopsis thaliana, Solanum dulcamara* and *Corchorus capsularis* demonstrated the considerable growth stimulating effects of pluronic (Poloxamer) co-polymers in both liquid and semi-solid systems. The possible mechanism(s) involved and their implications are considered in relation to the application of such compounds in plant biotechnology.

Kumar *et al.* (1991) reported that the non-ionic, copolymer surfactant, Pluronic F-68 (Poloxamer 188), is a valuable growth promoting supplements in plant culture systems. For example, addition of low concentrations of Pluronic F-68 to culture media stimulated growth of callus, isolated protoplasts and *Agrobacterium rhizogens*- transformed roots of *Solanum dulcamara*.

King *et al.* (1991) reported that related studies with animal cells have shown that Pluronic F-68 stimulates increased 2-deoxyglucose uptake and amino acid

incorporation into protein perhaps increasing cytoplasmic membrane permeability. This is supported by patch-clamp experiments using artificial lipid bilayers in which Pluronic F-68 caused the formation of short-lived, trans membrane pores. The inclusion of surfactants in plant culture media could prove beneficial, not only for stimulating tissue growth, but also in promoting differentiation.

2.2 *Agrobacterium* –mediated genetic transformation of white jute

2.2.1 Concept of genetic transformation

Genetic transformation of cells by uptaking of exogenous DNA has generated enormous interest in harnessing the advantages offered by plant tissue and cell culture technology. This consists of four steps: insertion, integration, expression and replication of foreign DNA inside the host cell. In all transformation experiments, specific reporter gene and one or more selectable marker gene are required to be incorporated into the plant cells prior to the integration of gene/ genes of interest. This reporter gene can be recognized in the plant tissue with the help of selectable agents, confirming transformation of the plant tissue (Gardner, 1993).

2.2.2 *Agrobacterium tumefaciens*- as a natural genetic engineer of dicots

Plant transformation mediated by *A. tumefaciens*, a soil plant pathogenic bacterium, has become the most useful method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors.

2.2.2.1 Biology of *Agrobacterium tumefaciens*

A. tumefaciens has the exceptional ability to transfer a particular DNA segment (T-DNA)of the tumor-induction (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing

crown gall disease (Nester *et al.*, 1984; Binns and Thomashaw, 1988). T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines.

These compounds produced by condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995).

Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crown gall and hairy roots respectively. These strains contain a large mega plasmid (more than 200 kb), which plays a key role in tumor induction and for this reason it was named Ti plasmid or Ri plasmid for *A. tumefaciens* in the case of *A. rhizogenes*. Ti or Ri plasmids are classified according to the T-DNA. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a cis element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the co-operative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The 30 kb virulence (*vir*) region is a regular organized in six operons that are essential for the T-DNA transfer (*vira*, *vir B*, *Vir D*, and *vir G*) or for the increasing of transfer efficiency (*virC* and *vir E*) (Hooykaas and Schilperoot, 1992; Zupan and Zambryski, 1995, Jeon *et al.*, 1998).

2.2.2.2 Important facts of *A. tumefaciens* transformation

The initial results of the studies on T-DNA transfer process to plant cells demonstrate three important facts for the practical use of the process in plants transformation. Firstly, the tumor formation is a transformation process of plant cells resulted from transfer and integration of T-DNA and subsequent expression of T-DNA genes. Secondly, the T-DNA genes are transcribed only in plant cells and do not play any role during the transfer process. Thirdly, any foreign DNA placed between the T-DNA borders can be transferred to the plant cells, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain system for plant transformation (for revive Hooykaas and Schilperoort, 1992; Deblaere *et al.*, 1985; Hamilton, 1997; Torisky *et al.*, 1997).

2.2.3 *Agrobacterium*-mediated transformation of crop plants

A. tumefaciens is a soil-dwelling bacterium and infect a wide range of dicotyledonous (and a few monocotyledonous) plant species. *A. tumefaciens* is the causative agent of crown gall disease, which is a plant tumor. This tumor inducing ability is based on a plasmid; Ti plasmid. This plasmid is the natural vectors for the delivery and insertion of foreign genes into plant DNA.

A number of useful markers for transformation and selection purposes are currently available. These are kanamycin, hygromycin and gentamycin. The ability of transformed cells to grow *in vitro* and initiate organs can differ widely. Usually, the frequency of recovery of transformants is much higher for kanamycin-supported selection and this marker has therefore been extensively used for many crops (Nehra *et al.*, 1990; James *et al.*, 1989). Another example is the neomycin phosphotransferase II (*npt*II) gene system. The NPTII protein catalyses the phosphorylation of kanamycin, neomycin and G418 with the phosphorylated antibiotics not being toxic to living cells. Plant cells or tissues transformed with this gene are therefore resistant to these antibiotics. Beside the

nptII gene, other marker gene i.e. the hygromycin gene (Severin *et al.*, 1989) and phosphinothricin acetyltransferase gene (Donn *et al.*, 1990) have also been used in plant transformation studies.

A number of tropical and sub-tropical crops are now amenable to gene transfer system by genetic engineering using *Agrobacterium*-mediated gene delivery system. A list has been compiled by Gardner (1993), Potato (De Block, 1988; Cardi *et al.*, 1992; Shahin and Simpson, 1986), citrus (Hidaka *et al.*, 1990), cotton (Umbeck *et al.* 1987), maize (Gould *et al.*, 1991), lettuce (Debnath *et al.*, 1995), jute (Hossain *et al.*, 1995), papaya (Manshardt, 1992), kiwifruit (Uematsu *et al.*, 1991, Rugini *et al.*, 1991; Janssen, 1991), strawberry (Nehra *et al.*, 1990), tobacco (An *et al.*, 1985), tomato (Fillati *et al.*, 1987; Mc Cormick *et al.*, 1986), amaranths (Pal and Chand, 1995), *Arabidopsis* (Nam *et al.*, 1997) and many other plants have been shown to be susceptible to *Agrobacterium*-mediated gene delivery system. However, the methods involved in genetic transformation are still developing and there are many plant species, which have not been transformed and regenerated to provide transgenic plants.

2. 2. 4 Genetic transformations in jute

During the last few years, substantial progress has been made in the development of transformation system of crop plants. Successful transfer of genes using *Agrobacterium* as a carrier has been achieved in a number of crops.

Khatun *et al.* (1990) and later in 1993 first reported genetic transformation of jute. Hypocotyls cotyledons of *C. capsularis* and *C. olitorius* were inoculated with *Agrobacterium rhizogenes* strains 8196 and A_4 T carrying wild Ri plasmids and with strains $p^{Bin\ 121}$, R1601 and LBA9402, p^{BIN19} carrying engineered plasmids. Explants were found to be susceptible to *A. rhizogenes* strains and produced roots from infected region. These roots were confirmed as transformed with positive opines and NPTII assays. Somatic embryos were obtained via callus formation

from transformed roots in the presence of 2, 4-D, but did not regenerate into plants.

Using a biolistic particle delivery system Ghosh *et al.* (2002) have developed an efficient protocol for the generation of stable genetic transformation in jute (*C. capsularis* var. JRC321). They used the apical, meristematic region of a germinating seedling as the explant. Transformation was carried out with the bialaphos resistance gene *bar* and the *rolC* gene of *A. rhizogenes*. The positive transformants containing the *bar* gene grew in the growth medium containing 2 mg/l bialaphos. Southern, Northern, Polymerase Chain Reaction (PCR) and reverse transcriptase-PCR analyses provided evidence of gene integration into the genomic DNA of jute. The T_0 transformants showed a stable inheritance of the gene to their progenies.

Haseena *et al.* (2000) reported *Agrobaterium*-mediated transformation for transfer of chitinase gene for conferring fungal resistance was attempted. Regeneration of jute plantlets transformed with marker genes and chitinase gene were grown in high selection pressure for two and seven months respectively. Transformed explants were repeatedly subculture in IAP-containing media to reduce the chimeric nature of regenerated jute plantlets from the multiple cells in the meristematic zone. With repeated subculture, at least 30% and 40% of the regenerating plantlets turned out to be albino in the 2 and 7 months old plantlets respectively.

Ahmed *et al.*, (1999) had made different attempts were made to develop *Agrobacterium*-mediated genetic transformation in jute. Two strains AGL1 and LBA4404 containing binary vectors pTab7 and pZ100 were used respectively. Cotyledonary bases with petiole from 48h old germinating seedlings were used as explants. In case of pTab7 2-3 putative transgenic leafed shoot lets were regenerated from the infected explants but were very weak and gradually died

within two months on the selection medium. In case of pZ100 different approaches like, co-cultivation and selection media variation, dark period duration, preculture of explants, no selection media for 10 days and alternate selection pressure were given. Though many shoots were regenerated in most of the cases but all of them become etiolated (white in color) and could not survive more than 6 weeks under selection pressure.

Gonggu (2000) reported that factors affecting differentiation of jute explants were investigated in China for developing an efficient transformation system for jute. In study 85% regeneration frequency was obtained. The results indicated that suitable explants were cotyledon any petiole and hypocotyls and differentiation can be promoted by pre-culturing of young explants for 24 h in medium with 8 mg/1 6-BAP. Shoot differentiation frequency can be improved by adding 20μM Acetosyringone to co-culturing medium and survival rate of explant was increased by addition of $AgNO_3$ in selection medium.

Gonggu *et al.* (2000) established an *Agrohucterium*-mediated gene transfer protocol after optimizing the factors affecting transformation in kenaf (*Hibiscus cannabinus* L.). The assay of GUS gene expression showed that Chitinase gene and *Bt* gene have been transferred into kenaf line 7804.

Khatun *et al.*(1994) reported multiple shoot-buds were regenerated from *Agrobacterium*-infected cotyledonary petioles of *C. capsularis*. Saha *et al.* (1996) and Islam *et al.* (1996) also reported an *Agrobacterium tumefaciens* based transformation system for jute following the plant regeneration techniques reported by Khatun *et al.* (1992).

However no further report was available on the survivability of the putative transgenic jute plants and in all these cases transformation of jute was not confirmed by DNA hybridization analysis.

Chapter 3
Materials and Methods

3.1 Experimental materials

3.1.1 Section-A. *In vitro* regeneration potentiality of *Corchorus capsularis* varieties

Varieties of *C. capsularis* such as CVE-3, CVL-1, D-154 and BJC-7370 were used in the present investigation to study different parameters associated with plant regeneration.

3.1.2 Section-B. *Agrobacterium*-mediated genetic transformation of *C. capsularis*

3.1.2.1 Plant materials

Two varieties of *C. capsularis* were used for plant transformation. The varieties were CVE-3 and CVL-1.

3.1.2.2 *Agrobacterium* strain and plasmids

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used for infection in the transformation experiment. This strain contains plasmid pBI121 of 14KDa (binary vector) (Fig.1). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct:

i. The udiA gene (Jefferson, 1986) encoding GUS (*β-glucuronidase*), driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation.

ii. The *nptII* gene (Herrera-Estrella *et al.*, 1983) encoding *neomycin phosphotransferase II* (*nptII*) conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid (132KDa) containing the virulence genes (Fig. 2).

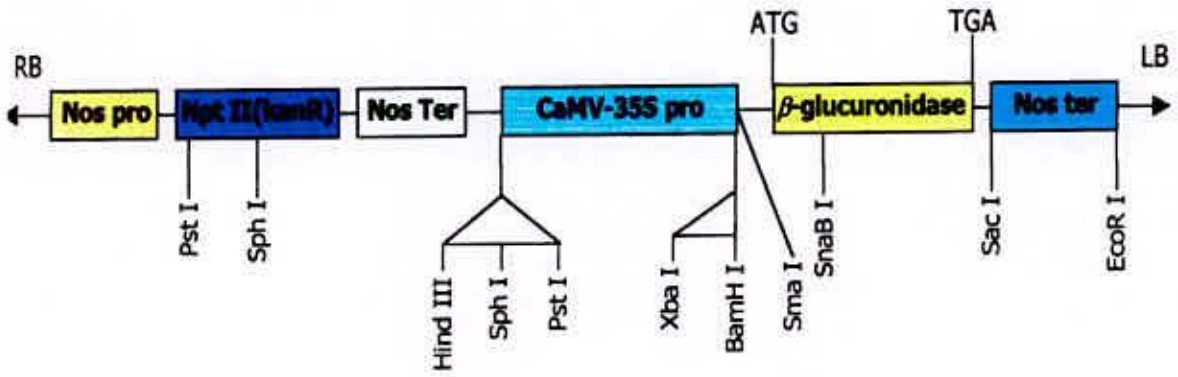


Fig. 1 pBI121 –Region between left border (LB) and right border (RB)

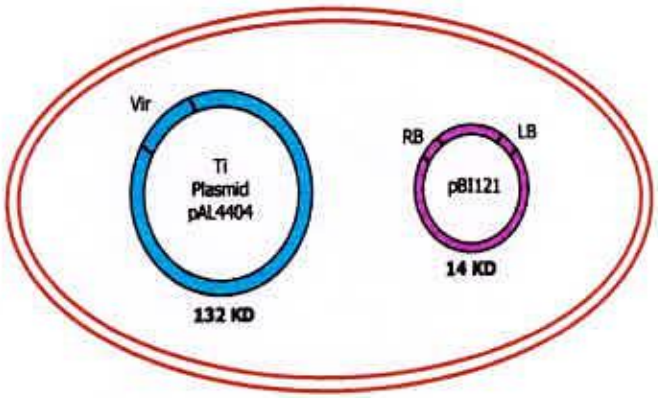


Fig. 2 *Agrobacterium tumefaciens* LBA4404

3.2 Sources of the experimental materials

Seeds of *C. capsularis* varieties used in this experiment were collected from Bangladesh Jute Research Institute (BJRI), Dhaka. The strain of *Agrobacterium tumefaciens* used in this study was obtained through the courtesy of Biochemistry and Molecular Biology Department, Dhaka University.

3.3 Location, time duration and year

To achieve the objectives, the experiments were conducted in the Genetic Engineering Laboratory, Genetic Resources and Seed Division, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from July 2006 to June 2007.

3.4 Media used

Different culture media used in the present investigation for various purposes were as follows:

3.4.1 Section-A

3.4.1.1 For seed germination

MS (Murasighe and Skoog, 1962) basal medium in combination with clinical cotton or agar.

3.4.1.2 For callus induction and shoot differentiation

i. MS medium as control

ii. MS medium supplemented by 2 mg/l BAP and 0.5 mg/l IAA.

3.4.2 Section-B

3.4.2.1 For seed germination

MS basal medium supplemented by clinical cotton.

3.4.2.2 For *Agrobacterium* culture and inoculation

YMB (Yeast extract and Mannitol Broth) medium was used with kanamycin Monosulphate as antibiotic for the maintenance of the strain LBA4404 of *A. tumefaciens*.

3.4.2.3 For co-cultivation

MS medium supplemented by 2 mg/l BAP and 0.5 mg/l IAA

3.4.2.4 For washing of explants after co-cultivation

MS liquid medium supplemented by 500-µg/ml cefotaxime

3.4.2.5 For selection and regeneration

MS medium supplemented by 2mg/l BAP and 0.5 mg/l IAA, kanamycin 50 mg/l and 500-µg/ml cefotaxime.

3.5 Methods

3.5.1 Preparation of culture media

For the induction of callus and plantlet regeneration in jute a number of culture media have been advocated by different scientists of which MS medium was used for investigating the present research work. A nutrient medium consist of organic and inorganic salts, irons, a carbon source, some vitamins and growth regulators were used. Composition of MS medium formulated by Murashige and Skoog, (1962) is given in Appendix I.

Different steps of media preparation are described below:

3.5.1.1 Preparation of stock solutions

The first requisite for preparation of medium was the preparation of stock solutions. Stock solution for growth regulators were prepared separately by dissolving the desired quantity of ingredients in appropriate solvent and the required final volume was made with water for ready use to expedite the preparation of the medium wherever needed. Separate stock solutions for macronutrients, micronutrients, iron, vitamins and growth regulators were prepared and stored appropriately for use.

i) Stock solution A (macronutrients): The stock solution for macronutrients was made up to 10 folds (10x) of the final strength of medium in 1000 ml of

distilled water. Ten times the weight of salts required per liter of the medium were weighed accurately and dissolved in 750 ml of distilled water and volume was made up to 1000 ml by further addition of distilled water. This stock solution was poured into a clean brown bottle, labeled with marker and stored in a refrigerator at 4°C for use.

ii) Stock solution B (micronutrients): This was made up to 100 folds (100x) of the final strength of the medium in 1000 ml distilled water (DW). The stock solution was labeled and stored in a refrigerator 4°C for later use.

iii) Stock C (Iron source): Required amount of $FeSO_4$ was added directly to the solution as powder.

iv) Stock solution D (Vitamins): Each of the desired ingredients except myo-inositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. Myo-inositol was used directly as powder at the time of media preparation.

v) Stock solution for hormones: Stock solution of hormones was prepared separately at 100 ml by dissolving the desired quantity of ingredients in appropriate solvent and the required volume was made with distilled water and stored in a refrigerator at 4°C for later use.

The following growth regulators (phytohormone supplements) were used in the present investigation.

Auxin: 3-indole acetic acid (IAA)

Cytokinins: 6-benzyl amino purine (BAP)

The growth regulators were dissolved in appropriate solvent as IAA in ethanol and BAP in 0.1N NaOH. For the preparation of stock solution of any of these hormones, 10mg of each of the hormone powder was taken on a clean beaker and dissolved in 1 ml of the appropriate solvent. The mixture was then collected in a 100 ml measuring cylinder and volume was made up to 100ml by the further addition of distilled water. The solution was then poured into a clean volumetric flask and stored at 4°C and used for maximum period of two weeks.

3.5.1.2 Steps followed for the preparation of culture media

In the course of present investigation, the following steps were followed for preparation of different culture media:

3.5.1.2.1 Preparation of MS medium

To prepare one liter (1000 ml) of MS medium, the following steps was followed:

- One hundred ml of macronutrients, 10 ml of micronutrients, .028 gm Iron and 10 ml of vitamins were taken from each of these stock solutions into a 2-liter Erlenmeyer flask on a magnetic stirrer.
- Four hundred and 50 ml distilled water was added in the flask to dissolve all the ingredients.
- Hundred mg of myo-inositol was added directly to the solution and dissolved well.
- Thirty grams of sucrose was added to this solution and agitated gently to dissolve completely.
- Different concentrations of hormone supplements were added to the solution either in single or in combinations as required and mixed well. MS not medium was prepared without hormone.

- P^H of the medium was adjusted to 5.8 with a digital p^H meter by adding NaOH or HCl (1% solution) whichever was necessary.
- The whole mixture was then made up to 500 ml with further addition of distilled water.
- 7.5 gm agar was dissolved in 500 ml distilled water and hot agar was added with rest 500 ml medium.

Required volume of hot medium was dispensed into culture vessels or conical flasks. After dispensing the medium the flasks were plugged with non-absorbent cotton plug and marked with different codes with the help of a permanent marker to indicate specific hormone combinations

3.5.1.2.2 Preparation of *Agrobacterium* culture medium

3.5.1.2.2.1 Preparation of YMB (Yeast extract and Mannitol Broth) medium for the maintenance *of Agrobacterium* strain LBA4404

For the growth of *A. tumefaciens* strain LBA4404, YMB medium was prepared in the following manner:

Component	Amount/100ml
Mannitol	1 gm
Yeast	0.04 gm
$MgSO_4.7H_2$	0.02 gm
NaCl	0.01 gm
KH_2PO_4	0.05 gm

The pH was adjusted to 7.0-7.2 before adding agar at 1.5gm. After autoclaving the medium was cooled to 50-55°C antibiotic kanamycin was added at a rate of 0.05 mg/ml and separated in petri dishes. When the medium became solid, the dishes were prepared for bacteria culture.

3.5.1.2.2.2 Preparation of YMB liquid medium for *Agrobacterium* suspension culture

For *Agrobacterium* suspension culture YMB liquid medium was prepared without agar. After autoclaving, the medium was cooled to 50-55^{0}C and 0.05 mg/l kanamycin was added.

3.5.1.2.2.3 Preparation of antibiotics (Kanamycin, and Cefotaxime)

Both the antibiotics are soluble in water.

- Required concentration of Kanamycin is 50 µg/ml
- Required concentration of Cefotaxime is 500 µg/ml

Preparation of stock solution of Kanamycin

Required concentration of Kanamycin 50 mg/l for this experiment to culture *Agrobacterium* and for use in selection media. Concentration of stock solution that prepared for stock was 50mg/ml and the total volume of stock solution 5 ml. So, 5 ml solution contains 50 mg x 5 = 250 mg kanamycin.

Steps:

- 250 mg kanamycin was weight by balance and taken in 5 ml. measuring flask.
- As kanamycin dissolves in water, 5 ml sterile distilled water was added in flask and dissolved by hand shaking.
- Filter sterilization was done with disposable filter sterilizer of 0.22 µm pore size and syringe.
- Distributed by 1 ml with the help of micropipette to five sterilized eppendrof tubes and stored at 4°C temperature.
- 1 ml stock contains 50 mg kanamycin.

Preparation of stock solution of Cefotaxime

Required concentration of Cefotaxime 500 mg/l for this experiment to control *Agrobacterium* in selection media and in washing solution. Concentration of stock solution that prepared for stock was 500mg/ml. Total volume of cefotaxime stock solution is 2 ml. So, 2 ml solution contains 500 mg x 2 = 1000mg or 1 gm cefotaxime.

Steps:

- Cefotaxime was supplied in 1gm vial in powder form.
- As it was dissolve in water, distilled water was sterilized by autoclaving.
- 2 ml sterile distilled water was injected into the cefotaxime vial through sterilized syringe and dissolved by hand shaking.
- The solution need not to filter sterilized and stored the vial at 4°C temperature.
- 1 ml stock contains 500 mg cefotaxime.

20(:) 03/04/08

3.5.1.2.2.4 GUS Histochemical Assay

3899/ 15.3.15

GUS histochemical assay can be done after co-cultivation of infected explants as well as after cultured on selection medium. Various β-D-glucuronic acid substrates are available for detection of GUS expression in *vivo* or n *vitro*. All of these substrates contain the sugar D-glucopyranosiduronic acid attached by glucosidic linkage to a hydroxyl group of a chromogenic, flurogenic, or other detectable molecule. The preferred substrate for GUS detection is 5-bromo-4-chloro-3-indolyl-β-D-glucuronide or X-gluc (Figure 3). This colorless substrate has high extinction coefficient (making it readily detectable at low concentrations) and aqueous insolubility of the final cleavage product, dichloro-dibromoindigo (ClBr-indigo).

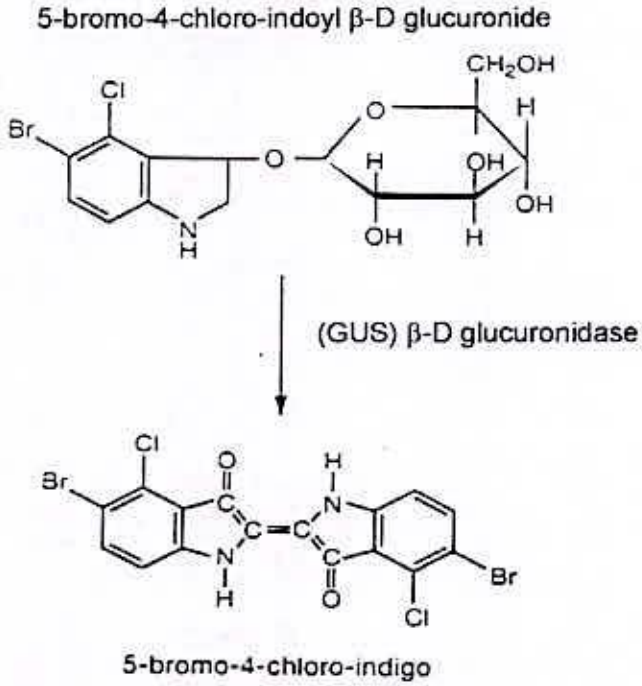


Figure 3: Reaction catalyzed by β-D glucuronidase

However, GUS staining solution is composed of following chemicals with their concentration.

Components	Amount/10 ml
X-gluc (solvent:DMSO)	8.89 mg
Chloramphenicol	1 mg
NaH_2PO_4	119.8 mg
Triton X (10%)	100µl
Methanol	2ml

pH was adjusted 7.0-8.0 by adding buffer pH-10 buffer solution up to 10 ml d.H_2O

3.5.1.2.2.5 Preparation of 10 ml GUS Staining Solution

Steps:

- All necessary glassware were autoclaved
- The 8.89mg X-gluc were weighted with the help of a digital balance and care should be taken that X-gluc have high molecular weight so very minute amount is required.
- Few drops of DMSO (Dimethyl Sulphoxide) were taken in a beaker and X-gluc was added.
- Gently shaken until all of the X-gluc was dissolve
- 200µl of Chloramphenicol was added in to the beaker.
- 10% Triton X was prepared by taken 20µl of Triton X into 200 µl of distilled. The Triton X first appeared a gel like semisolid substance, but soon dissolves if shaken gently. Then 100µl Triton X from this solution was added to the X-gluc solution.
- 2 ml of methanol was added to the solution and gently mix.
- pH of this solution was adjusted to 7.15 by adding pH buffer 10 solutions. It was noted that nearly 7 ml of buffer solution was need to adjust pH.

3.5.1.2.2.6 GUS (β-glucuronidase) Histochemical Assay Procedure

The co-cultivated calli, nodal segments and shoot tips were randomly selected from co-culture. The selected calli were cut into small pieces and were immersed in X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) solution and were incubated at 37^{0}C overnight. Similarly, the nodal segments and shoot tips were randomly selected from co-culture and were cut into pieces and sections. The pieces and sections were immersed in X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) solution were incubated at 37^{0}C overnight. The expression of GUS (β-glucuronidase) gene in the plant tissue was observed which showed blue color in the plant tissue. The transverse and longitudinal sections of nodal segments and part of shoot tips were observed under compound microscope and the photographs were taken with the help of digital camera.

3.5.2 Sterilization

To ensure aseptic condition in *in vitro*, all instruments, glassware and culture media were sterilized properly by autoclaving.

3.5.2.1 Sterilization of culture media

The conical flasks containing prepared media were autoclaved at 1.16 kg cm^{-2} pressure and 121^{0}C temperature for 20 minutes. For bacteria culture, YMB medium was then poured into sterile petri dishes in a laminar airflow cabinet and were allowed to cool before use.

3.5.2.2 Sterilization of glassware and instruments

Beakers, test tubes, conical flasks, pipettes, instruments like forceps, scalpels, inoculation loops, micropipette tips and eppendorf tubes were wrapped with aluminum foils. Empty flasks were capped with cotton plug and then were sterilized in an autoclave at a temperature of 121 $^{\circ}$C for 20 minutes at 1.16 kg cm^{-2} pressure.

3.5.2.3 Sterilization of culture room

The culture room was initially cleaned by gently washing all floors and walls with a detergent followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals. Generally, laminar airflow cabinet was sterilized by wiping the working surface with 70% ethyl alcohol.

3.5.2.4 Precautions to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet. The cabinet was switched on for at least half an hour before use and cleaned with absolute ethyl alcohol to overcome the surface contaminants. During the entire period of inoculation the autoclaved scalpels, forceps and inoculation loop were kept immersed into absolute alcohol contained in a glass jar inside the cabinet. At the time of inoculation these were again sterilized by flaming method inside the cabinet. Both the hands were rinsed with 70% alcohol. All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants.

3.5.3 Culture techniques (Section-A)

3.5.3.1 Experiment- 1. *In vitro* seed germination of *C. capsularis* varieties on agar supported and clinical cotton supported medium

Seeds of *C. capsularis* (vars. CVE-3, CVL-1, Tricap-2, D-154, Tricap-1 and BJC-7370) were surface sterilized by immersing in absolute alcohol for 1 minute and then in 0.1% (w/v) Mercuric Chloride for 20 minutes. Seeds were thoroughly washed with autoclaved distilled water for 6 times. The sterilized seeds were transferred in a 100 ml conical flask containing 50 ml of hormone free MS agar-solidified (0.8%, w/v) medium. Fifteen seeds were inoculated in each flask.

In another set of experiment, clinical cotton was used instead of agar as a supporting material for seed germination in association with MS basal medium. Clinical cotton was placed at the bottom of 100ml flasks. Each flask contained

20ml of hormone free MS liquid medium. Seeds of *C. capsularis* varieties were surface sterilized by immersing in absolute alcohol for 1 minute and then in 0.1% (w/v) Mercuric Chloride for 20 minute followed by 6 washes and placed on the surface of cotton-supported MS liquid medium. Cultures were placed in a growth room with 28^{0}C temperature under 1.0 Wm^{-2} of daylight fluorescent tubes with 12-hour photoperiod. Fifteen seeds were inoculated in each flask. Seven days old seedlings were used for further research work and data collection.

3.5.3.2 Experiment-2. Optimization of shoot regeneration in *C. capsularis* varieties at different BAP concentrations

Seeds were germinated on cotton supported liquid medium following the techniques described in Section 3.5.3.1 and cotyledons with attached petiole were used as explants. Ten explants were inoculated in each culture flask containing different treatments of BAP (0 mg/l, 1 mg/l, 2 mg/l, 3 mg/l, and 4 mg/l) and IAA (0.0 mg/l, 0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2 mg/l). The culture flasks containing explants were placed under fluorescent light in growth room with controlled temperature (28^{0}C). The flasks were checked daily to note the appearance of callus.

3.5.3.3 Experiment-3. Effect of different concentrations of $FeSO_4$ on shoot regeneration in *C. capsularis* varieties

The following culture techniques were employed in the present study:

a) Axenic culture

b) Explants culture

a) Axenic culture

Seeds were germinated on cotton supported liquid medium following the description in section 3.5.3.1. Seven days old seedlings were used as source of contamination-free explants.

b) Explants culture

The seedlings raised in axenic culture and explants were used as the source of explants. Twenty-four explants were inoculated in three replications containing different concentration of $FeSO_4$ (0, 28mg/l, 56mg/l, 84mg/l, and 112mg/l) supplemented with 2 mg/l BAP and 0.5 mg/l IAA. In Three replications containing explants were placed under fluorescent light in a growth room with controlled temperature (28^0 C). The flasks were checked daily to note the appearance of callus and shoot regeneration.

3.5.3.4 Experiment-4. Influence of surfactants (Pluronic F-68) on plant regeneration from cotyledon of *C. capsularis.*

The following culture techniques were employed in the present study:

a) Axenic culture

b) Explants culture

a) Axenic culture

Seeds were germinated on cotton supported liquid medium following the description in section 3.5.3.1. Seven days old seedlings were used as source of contamination-free explants.

b) Explants culture

The seedlings raised in axenic culture and explants were used as the source of explants. Twenty-four explants were inoculated in three replications of containing different concentration of surfactant% (0.005, 0.01, 0.02, 0.04, and 0.08) supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA. In three replication containing explants were placed under fluorescent light in a growth room with controlled temperature (28^0 C). The flasks were checked daily to note the appearance of callus and shoot regeneration.

3.5.4 Section-B *Agrobacterium*-mediated genetic transformation of *C. capsularis*

Genetic transformation is a powerful and important tool, can be used in plant breeding program for jute improvement as it permits access to an unlimited gene pool through the transfer of desirable genes from any source. But an efficient and reproducible transformation protocol is required for successful genetic transformation.

The following culture techniques were employed in the present investigation:

3.5.4.1 Axenic culture

Sterilized seeds were placed onto seed germination medium in 100ml conical flask following the techniques described in Section 3.5.3.1 In each flask 25-30 seeds were inoculated. The culture was then placed in a growth room. Seven days old seedlings were used as a source of contamination-free explants.

3.5.4.2 Explants preparation

The germinated seedlings raised in axenic culture were used as the source of explants. Cotyledonary petioles were used as explants. Seven days old cotyledons were excised from the seedlings.

3.5.4.3 *Agrobacterium* culture

A single colony from previously maintained *Agrobacterium* stocks was streaked into freshly prepared Petri-dish containing YMB agar-solidified medium having kanamycin. The Petri dishes were sealed with parafilm and kept in room temperature for at least 48 hours. This was then kept at 4°C to check over growth of the bacteria. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The cultures were sub-cultured regularly at each week in freshly prepared media to maintain the stock.

For infection, stock single colony of *Agrobacterium* strain was taken in an inoculation loop and inoculated in a conical flask containing liquid YMB medium with 50-mg/l kanamycin. The culture was allowed to grow at 28°C to get optimum population of *Agrobacterium* for infection and co-cultivation of explants.

3.5.4.4 Infection and incubation of explants

The *Agrobacterium* strain grown in liquid YMB medium was used for infection and incubation. To get suitable and sufficient infection of the explants, freshly excised explants were dipped into bacterial suspension for 1 minute before transferring them to co-cultivation medium.

3.5.4.5 Co-cultivation

Following infection and incubation, the explants were co-cultured on co-cultivation medium in petri dishes. Prior to transfer of all explants to co-cultivation media they were blotted dry with sterile filter papers for a short period to remove excess bacterial suspension. All the explants were maintained in co-cultivation media for one day. Petri dishes containing explants were placed under fluorescent illumination with 12 hours dark cycle at 28°C. The intensity of light was maintained at 1000 lux.

3.5.4.6 GUS histochemical assay

GUS activity was detected as described by Jefferson (1987). Randomly selected co-cultivated cotyledons cultured on selective medium were used for GUS assay. Immediately after inoculation on selection medium, cotyledons were incubated in GUS staining solution at 37^{0}C for 24 hours in darkness. The X-gluc was broken down by the activity of β-glucuronidase (GUS) gene, which was transferred with T-DNA in the cotyledonary tissue and produced a characteristic blue color.

3.6 Recording of data

To investigate the effect of different treatments and response of different varieties on seed germination, data were collected from the different parameter as given bellow:

a) Percent seed germination

The germination percentage was estimated as ratio of the number of seeds germinated to the number of seeds placed in the germination medium.

$$\text{Percent seed germination} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds placed in the medium}} \times 100$$

b) Per cent callus induction

Percentage callus induction was calculated on the basis of the number of explants placed and the number of calli induced.

$$\text{Percent callus induction} = \frac{\text{Number of explants induced calli}}{\text{Number of explants inoculated}} \times 100$$

c) Percent plant regeneration

The percentage of plant regeneration was calculated based on the number calli transferred to regeneration medium and the number of calli produced plantlets

$$\text{Percent shoot regeneration} = \frac{\text{Number of calli with plantlet}}{\text{Number of explants incubated}} \times 100$$

d) Average number of shoot per callus

Some calli produced only single shoot while some produced multiple shoots. Therefore, number of shoot per callus was recorded at 28 days interval and the mean was calculated using the following formula:

$$\overline{X} = \frac{\sum X_i}{n}$$

where,

$\overline{X}$ = mean of shoots/ca lus

$\sum$ = summation

X_i = number of shoots/callus

n = number of observation

3.7 Statistical analysis of data

The data for the characters under present study were statistically analyzed wherever applicable. The experiments were conducted in growth room and arranged in Completely Randomized Design (CRD) with five replications. The analysis of variance for different characters was performed and Duncan's Multiple Test (DMRT) compared means.

Chapter 4

Results and Discussion

4.1 Section-A. *In vitro* regeneration potentiality of *C. capsularis* varieties

4.1.1. Experiment-1. *In vitro* seed germination of *C. capsularis* varieties on agar supported and clinical cotton supported medium

Healthy seedling production was one of the major criteria for plant regeneration from jute explants. Seeds of C. *capsularis* varieties (CVE-3, CVL-1, D-154, and BJC-7370) were germinated on both agar solidified medium and surgical cotton supported liquid medium. Number of seeds germinated and percentage of seed germination were presented in Table-1.

4.1.1.1. Percent seed germination

4.1.1.1.1. Effect of varieties

There was significant effect of varieties in percent seed germination. The highest percentage of seed germination was found in variety CVE-3 (90.00%) and the lowest was found in BJC- 7370 (66.66%) Table-1.

4.1.1.1.2. Effect of media

Percent seed germination from the varieties *C. capsularis* was found to be higher on cotton supported liquid MS medium (85.33%) compared to agar solidified MS medium (62.16%). The result was shown in Table 2. Germination of jute seed and seedling growth in cotton supported liquid medium was found to be comparatively higher and healthier than agar-solidified medium. This finding is supported by the findings of Khatun (2001) who reported that germination percentage was higher in cotton-supported medium than the agar supported medium.

Table 1: Effect of different varieties on number of seeds germinated and percent of seed germination in cotton media

Variety	No. of germinated seeds/flask*	Percent of seed germination
CVE-3	13.50 a	90.00 a
CVL-1	12.50 ab	83.33 ab
Tricap-2	11.75 bc	78.33 bc
D-154	11.25 bcd	75.00 bcd
Tricap-1	10.50 cd	70.00 cd
BJC- 7370	10.00 d	66.66 d
CV (%)	9.19	9.19
LSD	1.604	10.70

Figures followed by same letter in a column do not differ significantly by DMRT.

* Each flask contain 15 seeds

Table 2: Effect of different media on number of seeds germinated and percent of seed germination

Media	No. of seeds germinated	Percent of seed germination
Agar	12.433 b	62.167 b
Cotton	17.067 a	85.333 a

Figures followed by same letter in a column do not differ significantly by DMRT.

4.1.1.1.3. Combined effect of varieties and media

The combined effect of varieties and media on percent seed germination has been presented in Table 3. The percent of seed germination was found to be the highest in cotton supported medium × CVE-3 (88.89%) and the lowest in agar supported medium × BJC-7370 (62.22%) (Plate 1 & 2). This finding is supported by the findings of Naher *et al.* (2003) who also found the highest in the variety CVE-3 (97.33%) in cotton supported medium. It might be concluded that cotton supported seed germination system was found comparatively better than agar supported system for the production of desirable explants.

Table 3: Combined effect of varieties and media on number of seeds germinated and percent of seeds germination

Treatment		No. of germinated seeds/flask*	Percent of seed germination
Variety	**Media**		
CVE-3	Agar	11.67 abc	77.77 abcd
	Cotton	13.33 a	88.89 a
CVL-1	Agar	10.33 abcd	68.88 bcd
	Cotton	13.00 ab	86.66 ab
D-154	Agar	9.667 cd	64.44 cd
	Cotton	12.33 abcd	82.22 abcd
BJC- 7370	Agar	9.33 d	62.22 d
	Cotton	11.00 abcd	73.33 abcd
CV%		13.77	13.69
LSD		2.624	17.39

Figures followed by same letter in a column do not differ significantly by DMRT.

* Each flask contain 15 seeds

A B

Plate 1. Seed germination of jute variety CVE-3 on culture media A. Clinical cotton-supported medium and B. Agar-supported medium

A B

Plate 2. Seed germination of jute variety BJC-7370 on culture media A Agar-supported medium and B Clinical cotton-supported medium.

4.1.2 Experiment - 2: Optimization of shoot regeneration in *C. capsularis* varieties at different BAP concentrations

In this experiment, different concentrations of BAP and constant IAA were used for shoot regeneration.

4.1.2.1 Effect of varieties

4.1.2.1.1 Average number of cotyledon regenerated

Significant variation was recorded among the different varieties of *C. capsularis* on consideration of average number of cotyledons regenerated (Table 4). The highest average numbers of cotyledons regenerated was recorded for the variety CVE-3 (4.40). On the other hand, the lowest number of cotyledons regenerated was counted in variety BJC-7370 (3.80).

4.1.2.1.2 Percentage of cotyledon producing shoots

Significant variation was recorded among the different varieties of *C .capsularis* on consideration of percentage cotyledons producing shoots (Table 4).The highest percentage cotyledons producing shoots was recorded for the variety CVE-3 (78.00%) .On the other hand, the lowest percentage cotyledons producing shoots was counted in variety BJC-7370 (60.35%).

4.1.2.1.3 Average number of shoots produced by each cotyledon

A statistically significant variation was recorded among different varieties of *C. capsularis* on consideration of average number of shoots produced by each cotyledon under the present experiment in laboratory condition (Table 4). The highest average numbers of shoots produced by each cotyledon was recorded for the variety CVE-3 (6.53). On the other hand, the lowest average numbers of shoots produced by each cotyledon was recorded in variety BJC-7370 (4.00).

4.1.2.2 Effect of different concentrations of BAP

4.1.2.2.1 Average number of cotyledon regenerated

Considering the average number of cotyledons regenerated, a statistically significant variation was found in different concentrations of BAP (Table 5). The highest numbers of cotyledons regenerated (4.50) were recorded in concentration of BAP 2 mg/l. On the other hand, the lowest number (1.78) was recorded in BAP concentration 4 mg/l.

4.1.2.2.2 Percentage of cotyledons producing shoots

A statistically significant variation was recorded in considering the percentage cotyledon producing shoots in different concentrations of BAP (Table 5). The highest percentage cotyledons producing shoots (80.00%) were recorded in BAP concentration 2 mg/l. On the other hand, the lowest percentage cotyledon producing shoots (30.00%) were recorded in concentration of BAP 4 mg/l.

4.1.2.2.3 Average number of shoots produced by each cotyledon

In consideration of the average number of shoots produced by each cotyledon, a statistically significant variation was found in different concentration of BAP (Table 5). The highest average numbers of shoots produced by each cotyledon (7.50) were recorded in BAP concentration 2 mg/l. On the other hand, the lowest average numbers of shoots produced by each cotyledon (1.50) were recorded in BAP concentration 4 mg/l.

Table 4. Percentage of shoot regeneration from the cotyledons (with attached petioles) of different varieties of *C. capsularis*

Variety	Number of explants produced shoots/flask*	Percent of shoots regeneration	Number of shoots produced by each cotyledon
CVE-3	4.40 a	78.00 a	6.533 a
CVL-1	4.00 b	69.02 b	4.032 b
BJC-7370	3.80 c	60.35 c	4.000 b

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

Table 5. Percentage of shoot regeneration from the cotyledons (with attached petioles) of different concentrations of BAP

Concentrations of BAP (mg/l)	Number of explants produced shoots/flask*	Percent of shoots regeneration	Number of shoots produced by each cotyledon
1.00	2.50 b	48.00 b	4.00 b
2.00	4.50 a	80.00 a	7.50 a
3.00	2.00 c	42.22 c	2.00 c
4.00	1.78 d	30.00 d	1.50 d

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

4.1.2.3 Combined effect of varieties and concentrations of BAP

4.1.2.3.1 Average number of cotyledon regenerated

The combined effect between varieties and concentrations of BAP also showed statistically significant differences in respect of average number of cotyledons regenerated under the present experiment. The highest number of cotyledon regenerated (5.00) was recorded in variety CVE-3 with concentration of BAP 2 mg/l. The lowest average number of cotyledons regenerated (1.33) was counted in variety BJC-7370 with concentration of BAP 4 mg/l. The details results are presented in Table 6.

4.1.2.3.2 Percentage of cotyledon producing shoots

The combined effect between varieties and concentration of BAP also demonstrated statistically significant differences in respect of percentage cotyledons producing shoots under the present trial. The highest percentage cotyledon producing shoots 83.33 %) was recorded in variety CVE-3 with concentration of BAP 2 mg/l (Table 6). The lowest average percentage cotyledons producing shoots (22.22%) was counted in variety BJC-7370 with concentration of BAP 4 mg/l (Plate 3 and 4).

4.1.2.3.3 Average number of shoots produced by each cotyledon

Combined effect between varieties and concentration of BAP also showed statistically significant differences in respect of average number of shoots produced by each cotyledon under the present trial in laboratory condition. The highest average number of shoots produced by each cotyledon (8.53) was recorded in variety CVE-3 with concentration of BAP 2 mg/l (Table 6). The lowest average number of shoots produced by each cotyledon (1.66) was counted in variety BJC-7370 with of BAP (4 mg/l).

Table 6. Percentage of shoot regeneration from the cotyledons (with attached petioles) of different varieties of *C. capsularis* and concentrations of BAP

Variety x concentration of BAP (mg/l)		Number of explants produced shoots/flask*	Percent of shoots regeneration	Number of shoots produced by each cotyledon
CVE-3	0	0.00	0.000	0.00
	1.00	3.00 c	50.00 c	4.27 c
	2.00	5.00 a	83.33 a	8.53 a
	3.00	2.66 cd	44.44 cd	2.63de
	4.00	2.00 def	33.33 def	1.83 e
CVL-1	0	0.00	0.00	0.00
	1.00	2.33 cde	38.89 cde	2.94 d
	2.00	4.33 ab	72.22 ab	8.16 a
	3.00	2.33cde	38.89 cde	2.16 de
	4.00	2.00 def	33.33 def	2.10 de
BJC-7370	0	0.00	0.00	0.00
	1.00	1.67 ef	27.78 ef	2.68 de
	2.00	4.00 b	66.67 b	7.16 b
	3.00	1.67 ef	27.78 ef	2.33 de
	4.00	1.33 f	22.22 f	1.67 e
CV%		18.58	18.57	18.56
LSD		0.6690	11.16	0.9622

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

Plate 3. Shoot regeneration in jute var. of CVE-3 on MS + 2 mg/l BAP + 0.5 mg/l IAA

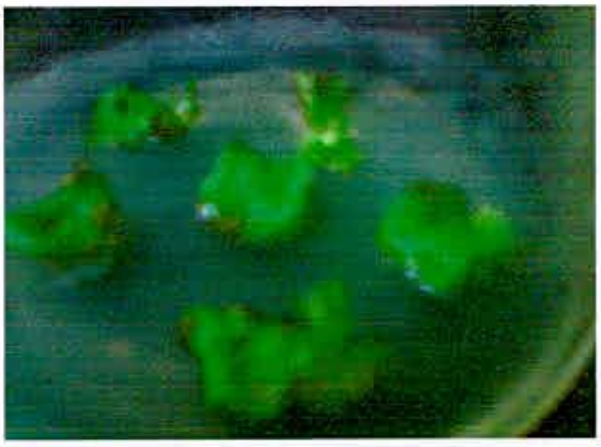


Plate 4. Shoot regeneration in jute var. of BJC-7370 on MS + 4 mg/l BAP + 0.5 mg/l IA

4.1.3 Experiment 3. Effect of different concentrations of $FeSO_4$ on shoot regeneration in *C. capsularis* varieties

To identify the optimum concentrations of $FeSO_4$ for regeneration of *C. capsularis* the cotyledons were cultured as explants in this experiment.

4.1.3.1 Main effect of varieties

4.1.3.1.1 Number of explants producing shoots

No significant variation was recorded among the different varieties of *C. capsularis* on consideration of number of explants producing shoots under the present experiment in laboratory condition. However, the highest number of explants producing shoot (5.47) regeneration was recorded for the variety CVE-3 and the lowest were counted in variety CVL-1 (5.33). The results are presented in Table 7.

4.1.3.1.2 Percent shoot regeneration

No significant variation was recorded among the different varieties of *C. capsularis* on consideration of percent shoot regeneration under the present experiment. (Table 7) However, the highest number of percent shoot regeneration (50.55%) were recorded for the variety CVE-3 and the lowest percent shoot regeneration were counted in variety CVL-1 (48.44%).

4.1.3.2 Effect of different concentration of $FeSO_4$

4.1.3.2.1 Number of explants showing shoots

A statistically significant variation was recorded in considering the number of explants showing shoot induction in different concentration of $FeSO_4$. The highest number of explants producing shoots (5.66) was recorded in concentration of $FeSO_4$ 28 mg/l. On the other hand the lowest number of explants producing shoot (3.33) was recorded in concentration of $FeSO_4$ 112 mg/l (Table 8).

Table 7. Effect of different varieties of *C. capsularis* on shoot regeneration

Varieties	Number of explants produced shoots/flask*	Percent of shoot regeneration	Number of shoots produced by each cotyledon
CVE-3	5.47 a	50.55 a	3.00 b
CVL-1	5.33 b	48.44 b	3.45 a

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

Table 8. Effect of different concentration of $FeSO_4$ of shoot regeneration on *C. capsularis.*

Concentration of FeSO4 (mg/l)	Number of explants produced shoots/flask*	Percent of shoot regeneration	Number of shoots produced by each cotyledon
0(control)	0.00	0.00	0.00
28	5.66 a	94.44 a	8.33a
56	5.33 b	88.89 b	6.67 b
84	4.00 c	66.67 c	5.83 c
112	3.33 d	55.56 d	4.33 d

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

4.1.3.2.2 Percent Shoot regeneration

A statistically significant variation was recorded in considering percent shoot regeneration in different concentration of $FeSO_4$. The highest percent shoot regeneration (94.44%) was recorded in concentration of $FeSO_4$ 28 mg/l. On the other hand, the lowest percent shoot regeneration (55.56%) was recorded in concentration of $FeSO_4$ 112 mg/l (Table 8).

4.1.3.2.3 Average number of shoots produced by each cotyledon

In consideration of the average number of shoots produced by each cotyledon, a statistically significant variation was found in different concentration of $FeSO_4$. The highest average numbers of shoots produced by each cotyledon (8.33) were recorded in $FeSO_4$ concentration 28 mg/l. On the other hand, the lowest average numbers of shoots produced by each cotyledon (4.33) were recorded in $FeSO_4$ concentration112 mg/l (Table 8).

4.1.3.3 Combined effect of varieties and different concentration of $FeSO_4$

4.1.3.3.1 Number of explants showing shoots

The combined effect of varieties and concentration of $FeSO_4$ also showed a statistically significant difference in respect of number of explants showing shoot under the present experiment. The highest number of explants showing shoot (5.66) was recorded in variety CVE-3 with concentration of $FeSO_4$ 28 mg/l (Table 9). The lowest number of explants showing shoot (1.66) was counted in variety CVL-1 with concentration of $FeSO_4$ 112 mg/l.

Table 9. Combined effect of different varieties of *C. capsularis* and Concentrations of $FeSO_4$ shoot regeneration

Varieties x concentration of $FeSO_4$ (mg/l)		Number of explants produced shoots/flask*	Percent of shoot regeneration	Number of shoots produced by each cotyledon
	0	0.00	0.00	0.00
	28	5.667 a	94.44 a	8.33 a
CVE-3	56	5.333 a	88.89 ab	6.66 bc
	84	4.000 cd	66.67 cd	5.83 c
	112	3.333 de	55.56 de	4.33 d
	0	0.00	0.00	0.00
	28	4.667 bc	77.78 bc	7.33 b
CVL-1	56	4.333 c	72.22 c	6.16 c
	84	2.667 e	44.44 e	6.11 c
	112	1.667 f	27.78 f	4.33 d
CV%		15.25	15.25	9.99
LSD		0.2787	13.81	0.8421

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

4.1.3.3.2 Percent Shoot regeneration

The combined effect of variety and concentration of $FeSO_4$ also showed a statistically significant difference in respect of percent of shoot regeneration under the present experiment. The highest percent shoot regeneration (94.44%) was recorded in variety CVE-3 with concentration of $FeSO_4$ (28 mg/l). The lowest number of percent shoot regeneration (27.78%) was counted for variety CVL-1 with concentration of $FeSO_4$ 112 mg/l (Table 9).

4.1.3.3.3 Average number of shoots produced by each cotyledon

Combined effect between varieties and concentration of $FeSO_4$ also showed statistically significant differences in respect of average number of shoots produced by each cotyledon under the present trial in laboratory condition. The highest average number of shoots produced by each cotyledon (8.33) was recorded in variety CVE-3 with concentration of $FeSO_4$ (28 mg/l) (Table 9). The lowest average number of shoots produced by each cotyledon (4.33) was counted in variety CVL-1 with concentration of $FeSO_4$ 112 mg/l Table 9 (Plate 5 and 6).

Plate 5. Shoot regeneration in jute var. CVE-3 on $FeSO_4$ (28 mg/l) concentration

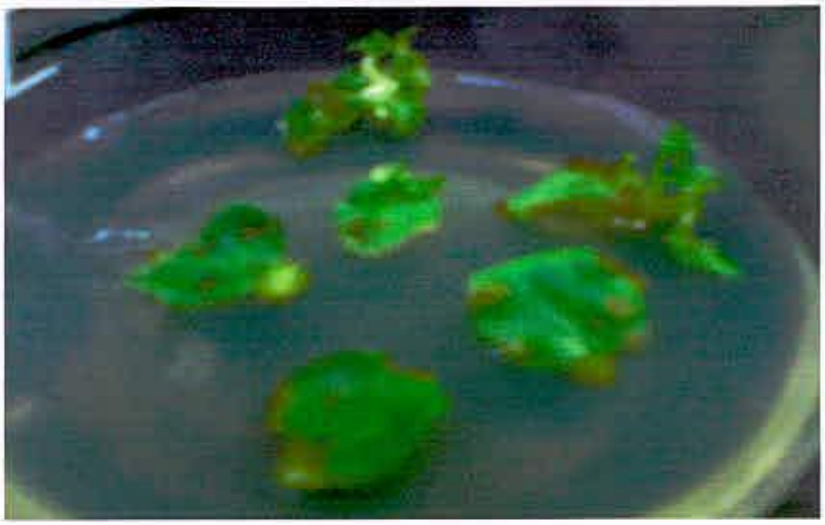


Plate 6. Shoot regeneration in jute var. CVL-1 on $FeSO_4$ (112 mg/l) concentration

4.1.4 Experiment-4. Influence of surfactants (Pluronic F-68) on plant regeneration from cotyledons of *C. capsularis*

4.1.4.1 Effect of varieties

There was significant effect of varieties in shoot regeneration and percent shoot regeneration. However there was no significant effect of number of shoot produced per cotyledon. The variety CVE-3 produced more cotyledon than other variety.

4.1.4.2 Effect of surfactant concentration

Mean value due to different concentration of surfactant for number of explants showing shoot, percent shoot regeneration and number of shoot, produced per cotyledon were significant (Appendix-v), indicating the presence of variation among the surfactant concentration used for the study, 0.08% was found the to be the best. Maximum number of explants produced shoot regeneration was (5.66) and percent shoot regeneration was (94.44) (Table-10).

4.1.4.3 Number of shoots per cotyledon

The combined effect of variety and surfactant for number of shoot per cotyledon was the highest in CVE-3 (7.66) at 0.04% surfactant and the lowest in CVL-1 at 0.02% surfactant. A lower shoot per cotyledons was noticed both at lower and higher surfactant concentration (Table 10).

4.1.4.4 Combined effect of varieties x Surfactant

The combined effect of varieties and surfactant (Table 10) showed that the highest shoot regeneration percentage in var. CVE-3 (94.44%) was recorded in MS media. The highest shoot regeneration percentage (88.89%) was recorded (var. CVL-1) in MS media supplemented with 0.08% surfactant followed by MS media with 0.04% surfactant (88.89%) (Plate 7 & 8). This finding is similar to the findings of

Khatun (1992) who found the best performance in shoot regeneration on the combination of MS media with 0.1% surfactant. It might be concluded that MS media with 0.1% surfactant combination is favorable for higher percentage of shoot regeneration; On the other hand, both lower and higher combination of surfactant reduced shoot regeneration. The results have been given in Table-10.

Table 10: Effect of different concentrations of surfactant (F-68) on number of explants showing shoot, percent of shoot regeneration and number of shoot produced per cotyledon

Varieties	Surfactant concentrations (%)	Number of explants produced shoot/flask*	Percent of shoot regeneration	Number of shoots/ cotyledon
CVE-3	0.0	3.33 c	55.55 c	4.06 c
	0.005	4.33 abc	72.22 abc	6.93 ab
	0.01	5.00 ab	83.33 ab	6.41 ab
	0.02	5.33 ab	88.89 ab	6.86 ab
	0.04	5.33 ab	88.89 ab	7.67 a
	0.08	5.67 a	94.44 a	6.72 ab
CVL-1	0.0	3.33 bc	55.55 c	3.63 c
	0.005	4.00 bc	66.66 bc	6.05 b
	0.01	4.33 abc	72.22 abc	6.06 b
	0.02	5.00 ab	83.33 ab	5.80 b
	0.04	5.33 ab	88.89 ab	6.60 ab
	0.08	5.33 ab	88.89 ab	6.26 b
CV(%)		15.70	15.70	11.55
LSD		1.248	20.80	1.191

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

Plate 7. Shoot regeneration in jute var. CVE-3 on 0.08% surfactant

Plate 8. Shoot regeneration in jute var. CVL-1 on 0.08% surfactant

4.2 Section-B. *Agrobacterium*-mediated genetic transformation of *C. capsularis*

In the present study, investigations were made to generate transgenic plants from two varieties of *C. caplusaris* (vars. CVE-3 and CVL-1) through *Agrobacterium* mediated transformation using cotyledons (with attached petiole) as explants.

4.2.1 Regeneration of putative transgenic shoots from *Agrobacterium* infected Cotyledons

Following infection and co-cultivation with the strain LBA4404, the cotyledons with attached petioles were cultured on plant regeneration medium containing cefotaxime 500 µg/ml for shoot development. Shoot regeneration from *Agrobacterium*-infected cotyledons was found to be the highest in CVE-3 (68.33%) followed by CVL-1 (43.33%) Table 11 (Plate 9 and 10). The average number of shoot produced by each cotyledon was also found the highest in CVE-3 (7.10) and the lowest in CVL-1 (5.15).

4.2.2 Histochemical GUS (β-glucuronidase) assay

After infection of the callus explants in *Agrobacterium* suspension culture, they were transfered to co-cultivation medium. Following incubation and co-cultivation with *Agrobacterium*, transformation ability was monitored through histochemical assay of GUS reporter gene in explants tissue. Transient GUS assay was done at the end of co-cultivation with randomly selected and inoculated explants tissue. In the GUS assay, conspicuous GUS positive (blue color) regions were detected in the explant surface (Plate.11 B and 12 B). The detailed results of the investigation were presented in Table 12.

Table 11. Effect of varieties of *C. capsularis* on percent shoot regeneration and average number of shoots from *Agrobacterium* infected cotyledon

Varieties	Number of explants produced shoots/flask*	Percent of shoot regeneration	Number of shoots produced by each cotyledon
CVE-3	4.10 a	68.33 a	7.101 a
CVL-1	2.60 b	43.33 b	5.158 b
CV%	14.93	14.92	5.66
LSD	0.50	8.43	0.35

Figures followed by same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 explants

Table 12. Effect of varieties towards GUS histochemical assay

Variety	Number of explants infected	Number of explants assayed for GUS	Number of explants +ve for GUS	% Of GUS +ve explants
CVE3	60	20	18	90.00
CVL1	60	20	15	75.00

Plate 9. Putative transgenic shoots of var. CVE-3 containing 50mg/l kanamycin on selection medium

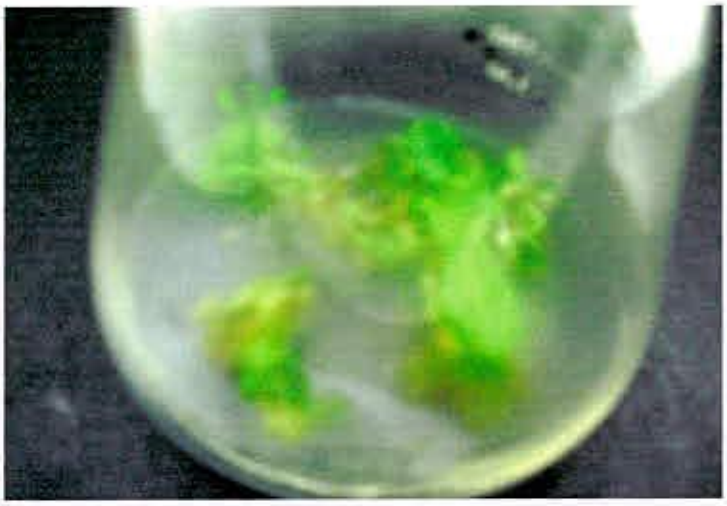


Plate 10. Putative transgenic shoots of var. CVL-1 containing 50mg/l kanamycin on selection medium

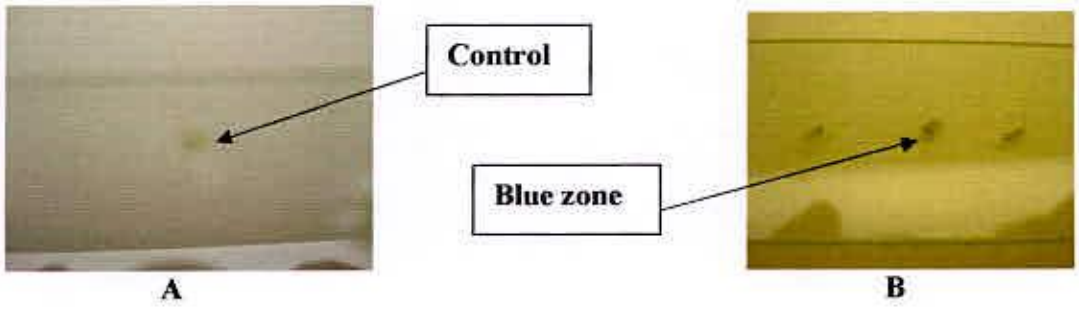


Plate 11. Histochemical localization of GUS activity (blue zone) at the infected cotyledonary tissue (B) with control explant (A)

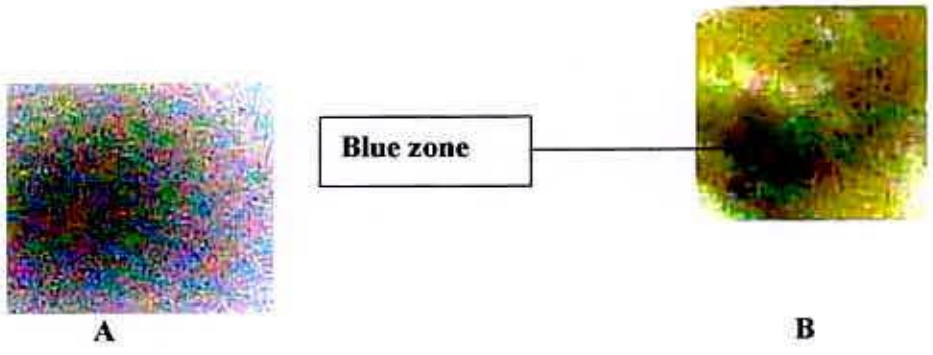


Plate 12. Magnified view of image A (11 A) and B (11 B)

Following GUS histochemical assay, it was found that the two varieties showed positive responses towards infection by *Agrobacterium*. Among the varieties, CVE-3 showed the highest (90.00%) and CVL-1 showed the lowest response (75.00%) to GUS assay (Table 12). Control explants did not show any response to the assay Plate 11 A and 12 A).

4.2.3. Selection of putative transformed cells and tissues

For selection of transformed cells and tissues, the callus proliferating shoots were transferred to selection and regeneration media containing 50mg/L kanakycin and 500 μg/ml cefotaxime. Presence of kanamycin in the selection media greatly affected the development of transgenic shoots (plate 13 and 14). Hossain *et al.* (1999) and Ahmed *et al.* (1999) also reported mortality of the transgenic plants six weeks after regeneration on selection medium.

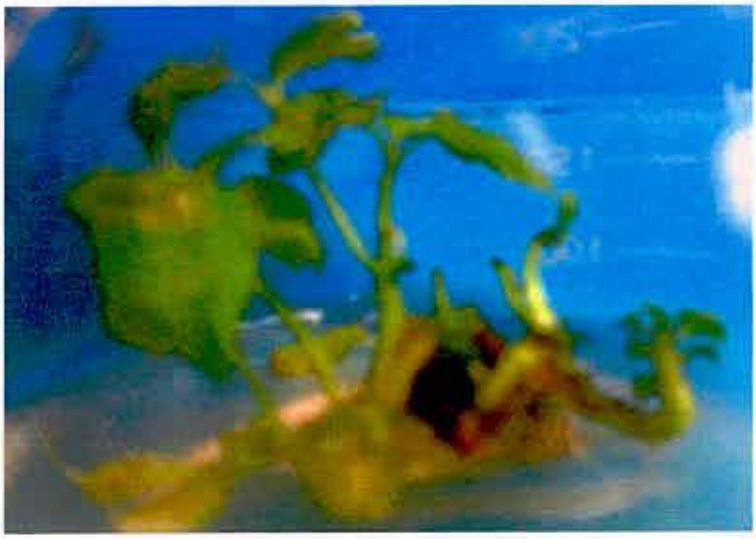


Plate 13. Putative transgenic plants of var. CVE-3 containing 50mg/l kanamycin on selection medium after two months

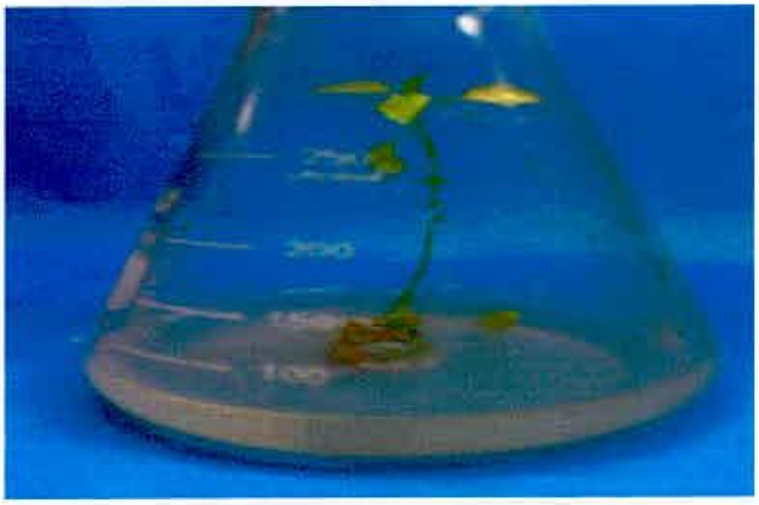


Plate 14. Putative transgenic plants of var. CVL-1 containing 50mg/l kanamycin on selection medium after two months

Chapter 5
Summary and Conclusion

Two different sets of experiments were carried out in the Genetic Engineering Laboratory, Genetic Resources and Seed Division, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from July 2006 to June 2007.

In the section-A, a detailed investigation was carried out to study the seed germination, callus induction ability and subsequent plant regeneration of *C. capsularis* genotypes using cotyledons (with attached petioles) as explants. In the section-B, investigation was carried out to study the *Agrobacterium* - mediated genetic transformation of white jute.

Significant effect of varieties was found in percent seed germination. The variety CVE-3 was found to be the highest in germination percentage (90%) and the lowest was found in BJC-7370 (66.66%). In cotton-supported medium, the germination percentage was found to be higher (85.33%) compared to agar solidified media (62.16%). From the results of the present study it was found that cotton supported medium performed better than agar-solidified medium for seed germination.

Cotyledons (with attached petiole) of three *C. capsularis* varieties were cultured on MS medium supplemented with 2mg/l BAP and 0.5mg/l IAA to observe their shoot regeneration capacity. A wide range of variation in shoot regeneration was exhibited by the varieties. The highest percent of shoot regeneration was found in CVE-3 (83.33%) and the lowest in Tri cap-2 (66.67%). No shoot regeneration was found without BAP and IAA. It was worth noting that percent shoot regeneration gradually increased with the increasing level of BAP up to 2mg/l. Further increase of BAP level did not show any improvement of shoot regeneration.

The varieties of *C. capsularis* (vars. CVE-3 and CVL-1) were cultured in different concentrations of $FeSO_4$ (0, 28mg/l, 56mg/l, 84 mg/l and 112mg/l) in association with MS plant regeneration medium. It was observed that CVE-3 showed relatively better shoot regeneration (94.44%) compared to CVL-1 (77.78%). $FeSO_4$ (28mg/l) showed best performance and maximum number of explants produced shoot regeneration in this concentration. The number of shoot per cotyledon was highest in variety CVE-3 (8.33).

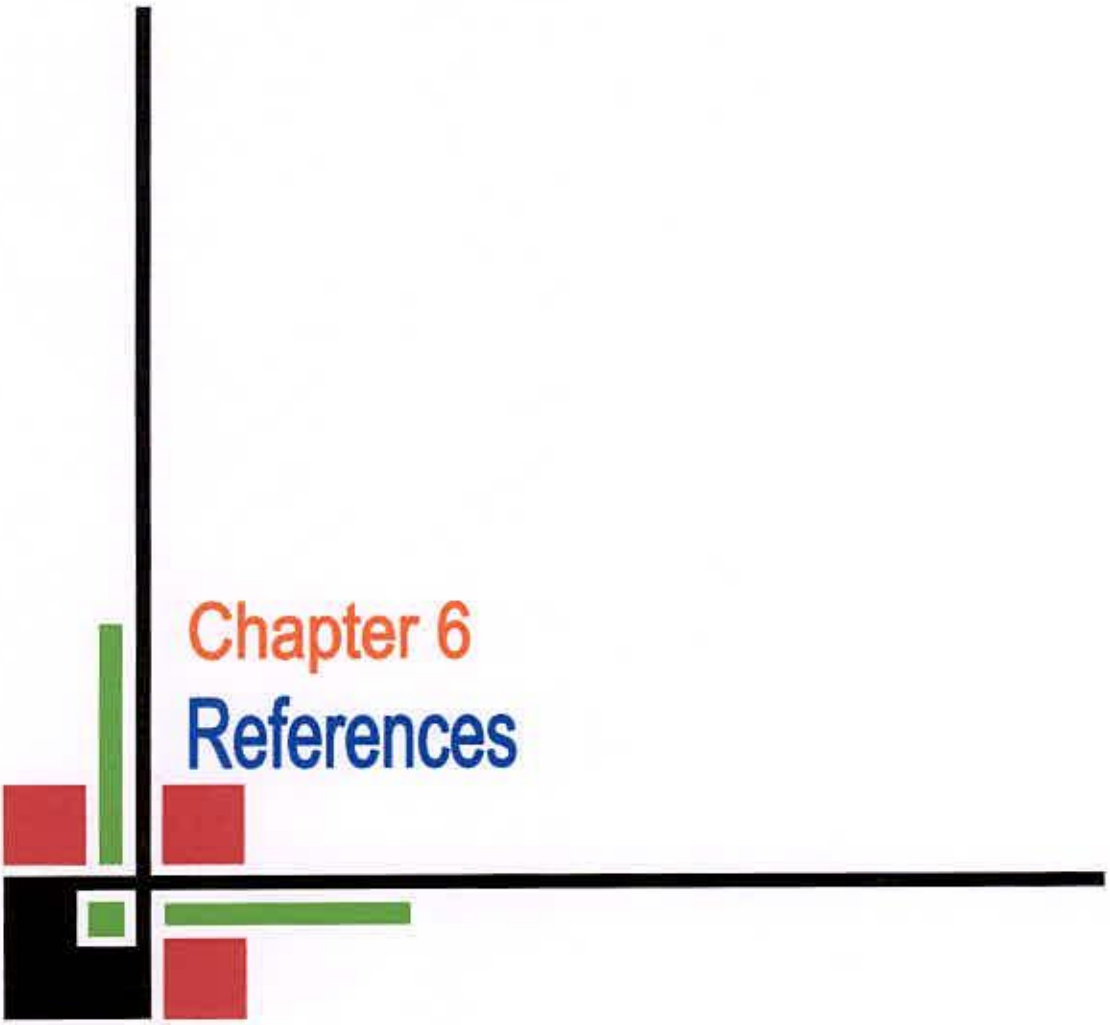
The varieties of *C. capsularis* (vars. CVE-3 and CVL-1) were cultured in different concentrations of surfactant in association with MS plant regeneration medium. It was observed that CVE-3 showed relatively better shoot regeneration (94.44%) compared to CVL-1 88.89%). Surfactant concentration at 0.08% was found to be the best. Maximum number of explants produced shoots regeneration in this concentration.

Following infection and co-cultivation, the explants were cultured on selection and regeneration media containing kanamycin along with cefotaxime and growth regulators. *Agrobacterium tumefaciens* strain LABA4404 has *nptII* gene within its T-DNA, which confers kanamycin resistance of transformed cells. Shoot regeneration from *Agrobacterium* infected cotyledons was found the highest in CVE-3 (68.33%). The putatative transformed regenerated plantlets grow on selection medium but died in course of time.

Histochemical GUS assay was performed soon after co-cultivation of explants with the bacterium. *Agrobacterium tumefaciens* strain LBA 4404 contains the GUS gene. This gene produces blue colour with X-gluc through GUS histochemical assay. Therefore, the presence of this blue colour successfully confirmed the integration of GUS gene from bacterial into the plant cell. Response of GUS assay was found to vary according to variety. Among the varieties, CVE-3 showed the highest response (90.00% GUS positive) and CVL-1 showed the lowest response (75.00% GUS positive) to GUS assay.

In the section-A, an efficient and reproducible protocol for the regeneration of *C. capsularis* genotypes has been developed using cotyledonary petioles as the explants. Since genetic engineering of crop plants relies on the development of efficient methods for the regeneration of viable shoots from cultured tissues, this protocol can be followed for genetic transformation of white jute.

An efficient protocol for the transformation of white jute varieties has been developed in the section-B, which showed the integration of two marker genes (GUS and *nptII*). Thus, in the future programme agronomically important gene/genes can be transferred to the white genotypes using this protocol. Particularly, for the development of disease and insect resistant white jute varieties this technique of transformation can be exploited successfully.



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Chapter 7
Appendices

APPENDICES

Appendix 1. Composition of MS medium (Murashige and Skoog, 1962)

a) Macronutrients	**Concentration (mgL^{-1})**
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
b) Micronutrients	**Concentration (mgL^{-1})**
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
KI	0.83
Na_2MoO_4	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Iron source	**Concentration (mgL^{-1})**
$FeSO_4.7H_2O$	27.80
Na_2-EDTA.$2H_2O$	37.30
d) Organic solvent	**Concentration (mgL^{-1})**
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine acid	0.50
Thymine	0.10
Myo-inositol	100

Appendix II. Analysis of variance (mean squares) for no. of seeds germinated and percent of seed germination

Source of variation	Degree of freedom	Characters	
		No. of seed germinated	Percent seed germination
Factor A(Variety)	5	6.129*	271.149*
Factor B (Media)	1	320.018*	8060.427*
Factor A x B (Variety x Media)	5	1.657 ns	41.417 ns
Error	48	2.402	105.480

* = 5% significant level of probability; ns = Non significant

Appendix III. Analysis of variance (mean squares) for average no. of cotyledons regenerated percent shoot regeneration and no. of shoots/cotyledon

Source of variation	Degree of freedom	Characters		
		Average no. of cotyledons regenerated	Percent shoot regeneration	No. of shoot/cotyledon
Treatment	14	6.946**	1929.442**	23.211**
Error	28	0.160	44.523	0.333

**= 1% significant level of probability

Appendix IV. Analysis of variance (mean squares) for no. of explants producing shoot, percent shoot regeneration and no. of shoots/cotyledon

Source of variation	Degree of freedom	Characters		
		No. of explants producing shoot	Percent shoot regeneration	No. of shoot/cotyledon
Treatment	9	12.611**	3503.56**	24.471**
Error	18	0.233	64.812	0.241

**= 1% significant level of probability

Appendix V. Analysis of variance (mean squares) for no. of explants producing shoot, percent shoot regeneration and no. of shoots/ cotyledon

Source of variation	Degree of freedom	Characters		
		No. of explants producing shoot	Percent shoot regeneration	No. of shoot/cotyledon
Treatment	11	1.967**	546.469**	4.034**
Error	22	0.543	150.838	0.495

**= 1% significant level of probability

Appendix VI. Analysis of variance (mean squares) for no. of explants producing shoot, percent shoot regeneration and no. of shoots/cotyledon

Source of variation	Degree of freedom	Characters		
		No. of explants producing shoot	Percent shoot regeneration	No. of shoot/cotyledon
Treatment	1	11.250**	3124.750**	18.876**
Error	9	0.250	69.435	0.120

**= 1% significant level of probability