PROTOCOL DEVELOPMENT FOR GENETIC TRANSFORMATION IN *Corchorus olitorius* THROUGH *Agrobacterium* VECTORS

BY

MIR MD. SHAHIN AKTAR

REGISTRATION NO. 26189/00483

A Thesis
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

SEMESTER: JANUARY-JUNE, 2007

Approved by:

.....
(Dr. Asma Khatun)
Chief Scientific Officer
Genetic Resources and Seed Division
BJRI, Dhaka
Supervisor

.....
(Dr. Md. Sarowar Hossain)
Professor
Co-supervisor

.....
(Professor Abu Akbar Mia)
Chairman
Examination Committee

GENETIC RESOURCES AND SEED DIVISION
Bangladesh Jute Research Institute (BJRI)
Manik Mia Avenue, Dhaka-1207
Telephone: 9145933; Email: asmak@bttb.net.bd

CERTIFICATE

This is to certify that the thesis entitled, "Protocol Development for Genetic Transformation in Corchorus olitorius Through Agrobacterium Vectors" 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 bonafide research work carried out by MIR MD. SHAHIN AKTAR Registration No. 26189/00483 under my supervision and my 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.

Asma Khatun

.....

(Dr. Asma Khatun)
Supervisor
Chief Scientific Officer
Genetic Resources And Seed Division
BJRI, Dhaka

Dated: June, 2007
Place: Dhaka, Bangladesh

Dedicated To My Beloved Parents

Dedicated To My Beloved Parents

LIST OF ABBREVIATIONS

Abbreviations		Full Words
%	:	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
dw	:	Distilled Water
e.g.	:	Exempli gratia (by way of example)
et al.	:	et alu=other people
etc.	:	et cetera (means and the rest)
g	:	Gram
gl^{-1}	:	Gram per litre
GUS	:	B-glucuronidase
HCl	:	Hydrocloric acid
$HgCl_2$	:	Mercuric Cloride
hrs.	:	Hours
i.e.	:	ed est (means That is)
IARI	:	Indian Agricultural Research Institute
ICRISAT	:	International Crop Research Institute fo· the Semi-arid Tropics
IRRI	:	International Rice Research Institute
j.	:	Journal
Kan	:	Kanamycin
LB	:	Luria Broth
mgl^{-1}	:	Milligram per litre
ml	:	Millititre
MS	:	Murashige and Skoog
Na_2-EDTA	:	Sodium salt of ferric ethylene di-amine tetra-acetate
NAA	:	α-napthalene acetic acid
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
No.	:	Number
nptII	:	Neomycin phosphotransferase II
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

Abbreviations		Full Words
via	:	By way of
Vir	:	Virulence region
viz.	:	Namely
X-Gluc	:	5-bromo-5-chloro-3-indolyl glucoronide
YMB	:	Yeast extract Mannitol Broth
µg	:	Microgram
>	:	Less than
<	:	Greater than
µl	:	Micro letter(s)

ACKNOWLEDGEMENTS

All praises, gratitude and thanks are due to Almighty "Allah", who enabled the author to complete the research work and thesis writing leading to Master of Science (MS) in Genetics and Plant Breeding.

I have the great pleasure in expression my deepest sense of gratitude to Dr. Asma Khatun, ,Chief Scientific Officer, Genetic Resources and Seed Division, BJRI, Dhaka for suggestion of the thesis topic, inspiring guidance, scholarly suggestions, constructive criticism and constant encouragement throughout the course of the investigation and spending valuable time to go through the manuscript. I owe a lot to her for sustained interest and tireless help and no amount of rhetoric would suffice to express my indebtedness to her.

I am highly grateful to Professor Dr. Md. Sarowar Hossain, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University of my Co-supervisor for his support and encouragements during the course of my studies and for providing unforgettable help at the time of exigency.

It gives me immense pleasure to express my profound sense of gratitude and sincere thanks to Professor Abu Akbar Mia, Chairman, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University for the precious encouragement, constructive suggestions, invaluable guidance, expert evaluation and keen interest that helped me to overcome every problem that came to my way during the course of this investigation and preparation of the manuscript.

Sincere thanks and profound gratitude extended to all teachers of the Department of Genetics and Plant Breeding for their valuable advice, inspiration and all possible help during the whole period of my study.

The author expresses his special note of thanks and gratitude to his honourable teacher Dr. Amzad Hossain, PSO, BSRI, Iswardi, Pabna for his valuable advice, inspiration and all possible help during Gus analysis.

I express my heartiest and esteem sense of gratitude to Md. Mostofa Kamal, SSO and Chandan Kumar Shaha, PSO, BJRI for their suggestion and valuable guidance at all stage of my research.

I take this opportunity to thank the technical staff of BJRI, for help and recording necessary observations for my study and stay.

I would like to express heartiest thanks to my friends for their support, co-operation, kind help and above all for their lovely company during my study.

I am immensely indebted to the love and courage bestowed upon my mother, brothers and sister for their sacrifice and devotion. Blessing of my beloved father inspired me to complete this work. Also, my sincere thanks to my relatives for their moral support, blessings and constant encouragement without which I would not have complete this endeavour. I remember them whole heartedly.

There are many others who, in various ways helped and supported me. I sincerely thank all of them and request their forgiveness for not mentioning them here by name.

Dated: June, 2007
Place: SAU, Dhaka.

The Author

CONTENTS

CONTENTS CONTD.

CONTENTS CONTD.

CONTENTS CONTD.

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

PROTOCOL DEVELOPMENT FOR GENETIC TRANSFORMATION IN *Corchorus olitorius* THROUGH *Agrobacterium* VECTORS

BY

MIR MD. SHAHIN AKTAR

ABSTRACT

Two sets of the experiments were conducted at the Genetic Engineering Laboratory, Genetic Resources and Seed Division, Bangladesh Jute Research Institute (BJRI), Dhaka, during the period of July 2006 to June 2007, to fulfill the objectives of the study. In the first set of the experiments, two varieties of *C. olitorius* were used to investigate their *in vitro* regeneration potentiality, and in the second set of the experiments, two varieties of *C. olitorius* were used to observe their transformation ability. Cotyledons (with attached petiole) were used as explants. The percentage of germinated seeds (90.00%) on cotton-supported medium found to be much higher than germinated seeds (68.33%) on agar-supported medium. The seedlings grown on cotton-supported medium found to be much more healthier than seedlings grown on agar-supported medium. Maximum no. of plant regeneration percent was obtained from the cotyledonary petioles of the varieties of *C. olitorius* on MS agar solidified medium supplemented with 0.5 mg/l IAA and 3.0 mg/l BAP. Plant regeneration was also observed at different concentrations of $FeSO_4$ and it was found that, 56 mg/l gave the highest result. The efficiency of plant regeneration from the cotton-supported seedlings was better than the agar-supported seedlings. The plantlets produced roots on hormone free MS medium rapidly. The effects of non–ionic surfactants (Pluronic F-68) on shoot regeneration for jute (*C. olitorius*) cotyledons was assessed. Supplementation of culture media with 0.01-0.08% (w/v) Pluronic F-68 increased the

mean percentage of cotyledon producing shoots. In the second set of the experiments, an efficient and reproducible protocol for the production of transgenic jute plant was developed by inoculating cotyledonary petioles with *A. 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 (O-9897 and O-72). In the transformed explants, GUS reporter gene was expressed showing blue colour in the explant tissues. Non-transformed explants did not show any colour. Between the varieties, O-9897 showed the highest response to GUS assay (60.00%).

CHAPTER-1

INTRODUCTION

INTRODUCTION

Jute constitutes a major world fiber crops, which is particularly important to the economy of Bangladesh. The two cultivated species *C. capsularis* and *C. olitorius* (2n=14), belonging to the family of Tiliaceae yield a bast (bark) fiber which is one of the most important vegetative fiber next to cotton (BJRI, Annual Report, 2003-2004). Commonly *C. olitorius* is known as tossa jute and *C. capsularis* as white jute. Jute is not only a major foreign currency earner, but also a major source of employment for the producer countries. 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, 2005). 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 2003-2004, the acreage, production and yield of jute was 1128 thousand acre, 859 thousand bales and 762 kg/acre respectively (BBS, 2005). It is extensively used in the manufacture of different types of packing materials for various agricultural and industrial products.

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 2004-2005 Bangladesh exported 619000 tons of raw jute and jute goods and earned about 16908 million Taka. (BBS, 2005).

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 lakh 67 thousand bales of jute are damaged by insect-pest (Ahmed *et al.* 1993). Diseases have also an adverse effect on yield. A biotic stresses like drought, flood and low temperature are detrimental

to this crop. With the launching of global campaign for environmental awareness international 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. Sustainable improvement in jute productivity under less favorable environment can only be achieved with a constant flow of new genetic materials. The existing variable for the constraints of jute productions are insect-pest, diseases, poor soil fertility, water stress, fiber quality and photo-insensitive. One of the major constraints to increase jute productivity is the non-availability of modern varieties with improved plant types.

Modern biotechnological techniques may also be utilized conveniently to overcome the constraints related to jute improvement and production. Genetic transformation is one of way by which foreign gene can be transfer to any specific genome. The transformation technique to higher plants has been accomplished by different methods (Gardner 1993, Paszkowski *et al.*, 1989). The most common and efficient one utilizes non-cogenic *Agrobacterium* strain as a gene vector. It is evident that all the tissue culture techniques play a vital role in the enrichment of genetic variability. It can contribute to produce disease and pest resistant transgenic jute for higher production.

Although considerable number of high yielding varieties of jute has been released from the Bangladesh Jute Research Institute (BJRI) through conventional breeding techniques, these techniques still have some limitations. It is therefore, very important to explore other means of modern scientific techniques for example, tissue culture or genetic engineering to accelerate the pace of varietal 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. Biotechnological approach can utilize for the same. The pre-requisite for the genetic transformation in jute is to establish an efficient system of plant regeneration. Plant regeneration from the cotyledon petioles reported earlier in *C. capsularis* (Khatun *et al.*, 1992) and also from the shoot apices of *C. olitorius* (Khatun *et al.* 2001-2002). Biotechnological approaches for crop improvement is a

new research area in Bangladesh. Research on plant biotechnology in bast fiber crops especially on jute has been conducted in few laboratories of Bangladesh. Some achievements have been made regarding tissue and cell culture techniques.

Jute is susceptible to root-knot nematodes and spiral borers. It is also susceptible to stem rot and leaf mosaic diseases. Resistant genes are available against stem borer, fungus and viral diseases which can be inserted in jute through genetic transformation technique. The developed protocol for plant regeneration from jute would be used for insertion of agronomically important genes in jute plants. Marker genes e.g. kanamycin and GUS genes would be used for jute transformation.

With this view in mind, the present research work has been undertaken for the following objectives:

i. To establish an efficient and repeatable plant regeneration protocol for jute plant.

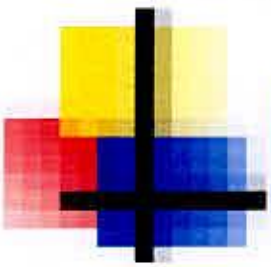
ii. To find out the optimization of shoot regeneration at different concentrations of BAP

ii. To know the effects of $FeSO_4$ on shoot regeneration.

iii. Optimization of shoot regeneration from the explants of *C. olitorius* varieties at different concentrations of surfactants (Pluronic F-68).

iv. Protocol development for the insertion of a foreign gene in jute plant through *Agrobacterium* vectors.

CHAPTER-2



REVIEW OF LITERATURE

REVIEW OF LITERATURE

Jute is the most important fiber crop in Bangladesh more attention is needed for research on production, utilization and improvement of jute plant. Improvement of jute like other crop through conventional method requires long time. Modern advances in tissue culture and recombinant DNA technology have opened new opportunity in transformation and improvement of higher plants, which accordingly produced many transgenic plants with new genetic properties. Establishment of an efficient plant regeneration system from the jute explants is a prerequisite to create variability and to introduce foreign genes into this crop through genetic transformation (Khatun, 1993). Nevertheless, some of the important and informative works and findings so far been done in Bangladesh and abroad on plant regeneration and transformation of jute and other allied fiber crops have been reviewed in this chapter.

2.1.1 Concept of tissue culture

Conventional techniques for crop improvement are prolonged processes and take more time for crop improvement. The techniques of plant tissue culture have been developed as a new and powerful tool for crop improvement (Carlson, 1975) and received wide attention of modern scientists (D'Aamato1978, Skirvin 1978). Now a days, plant tissue culture techniques have been come forward as a world wide accepted concept (Hoque, 2001) and opened up several new avenues for manipulation of crop plants to induce genetic changes and selection of desirable traits (Nath, 2001). Besides, plant regeneration from *in vitro* cultures is a prerequisite of genetic transformation techniques in many crop plants (Akter, 2001).

2.1.2 Tissue culture of jute

Tissue culture technique is now used extensively in many national and international organizations, such as BJRI, BSRI, CIP, IARI, ICRISAT and USDA, where different crop improvement programs are in progress for development of different crop varieties.

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

Islam (1981) obtained callus initiation and root formation from explants of *C. olitorius* and claimed to have obtained a few shoots directly from leaf explants of *C. olitorius* on MS medium. Plant regeneration of jute from meristem (Rahman *et al.*, 1985), cotyledon (Rahman *et al.*, 1985; Khatun *et al.*, 1992; Ali, 1992), leaf (Islam, 1981), plumule (Das *et al.*, 1986), hypocotyl (Khatun *et al.*, 1992; Ghosh and Chatterjee, 1990; Seraj, 1992), apical meriatems (Khatun, 1993) and anther culture (Islam, 1981) have been reported. Tissue culture research in jute was started in 1964, when Islam cultured inter-specific hybrid embryo (*C. capsularis* × *C. olitorius*) and hybrid plants were obtained. *In vitro* regeneration has been reported to be quite difficult among the species of *Corchorus* through tissue culture technique (Khatun, 1993).

2.1.3 *In vitro* seed germination

Healthy seedling production was found to be 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:

Khatun (1993) conceded out an experiment to study the germination percentage of different varieties of *C. olitorius* namely vars. O-9897, O-72, OM-1, O-4 on hormone free agar-solidified MS basal media and cotton-supported MS liquid media. She observed that the germination percentage of the varieties was found higher on cotton-supported medium than the agar medium.

Khatun (2003) also renowned that the highest germination percentage was found in the var. O-9897 (98.66%) on cotton-supported medium and the lowest germination percentage was observed in the var. O-72 (38.66%) on agar medium.

2.1.4 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 varieties in the combinations of growth regulators were reported by several workers. The most relevant literatures related to callus induction have been reviewed here.

2.1.5 Varietal difference

Murashige and Skoog (1962) reported that nutritional requirements for optimal growth of tissue *in vitro* may vary with varieties. Even tissue culture of different parts of a plant may show different requirements for satisfactory growth.

Khatun (2003) conducted an experiment on four varieties of jute (vars. O-9897, O-72, OM-1 and O-4) and observed that the frequency of shoot production varied greatly among the varieties. She reported that var. O-9897 showed the best performance in shoot regeneration. Two species of *Corchorus* were tested for plant regeneration (Khatun, 1993) and she observed that plant regeneration from the explants of *C. olitorius* was very different in culture condition than *C. capsularis*. She made several attempts by using various hormone and media combinations *in vitro* to obtain plant regeneration from various explants sources of *C. olitorius*.

2.1.6 Effects of explants

Rahman *et al.* (1985) reported that callus initiated from both apical meristems and cotyledons of the var. O-9897 of *C. olitorius*, when cultured on BAP and tyrosine fortified on MS media and finally formed shoot. Ghosh and Chatterjee (1990) reported plant regeneration from hypocotyl derived callus tissue on MS medium in *C. olitorius*. Khatun *et al.* (1992) reported about cotyledon derived callus. They used phytohormones BAP and IAA with MS medium to develop multiple shoots from cotyledon derived calli. Ali (1992) also reported similar results from another experiment. Tewari *et al.* (1999) reported that 2, 4-D induced callus in 100% of explants when cotyledons, segments of hypocotyls and roots of white Jute (*C. olitorius)* were culturred on MS medium supplemented with 16 adjuvant individually and/or in combination. Plant regeneration from the explants (cotyledon segments, hypocotyl segments and root segments) of *C. olitorius* (vars. O-9898, OM-1, O-72 and O-4) was difficult. However, the explants of cotyledonary petioles of all these varieties produced shoots from the cut ends (Khatun, 2003).

2.1.7 Maintenance of callus

Very little work and attention has been paid so far on maintenance of callus of jute. Therefore, maintenance of jute and other crops have been reviewed. The organogenic callus of *C. olitorius* (vars. O-9897 and O-72) were found rich in large starch granules, was transferred to MS basal medium, and it differentiated into single or multiple shoots (Seraj *et al.*, 1992). A long-term regeneration system for garlic clones was developed by Myers and Simon (1999) where callus was initiated on modified Gamborg's B-5 medium supplemented with 4.5 µM 2, 4-D and maintained on the same basal medium with 4.7 µM piclorum and 0.4 µM 2ip (isopentenyladenine). Regeneration potential of callus after 5, 12 and 16 months on maintenance medium was measured using several plant growth regulator treatment.

2.1.8 Effects of growth regulators

Tewari *et al.* (1999) reported that MS medium supplemented with 2, 4-D induced 100% calli production in jute.

Khatun (2003) 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 concentrations of BAP (2, 3, 4 or 5 mg/l) and she observed the best performance in shoot regeneration on the combination of MS + 0.5 mg/l IAA and 2 mg/l BAP.

2.2.1 Effects of surfactants (Pluronic F-68) on shoot regeneration

The marked lack of response of *C. olitorius* cotyledons to Pluronic F-68 supplementation probably reflects reduced morphogenic capacity of this species compared to *C. capsularis* (Rahman *et al.*1985). Thus, it appears that jute cotyledons will only response to Pluronic F-68 stimulation provided that they have a regeneration response to exogenously supplied plant growth regulators. In this regard, it should be pointed that presence of Pluronic F-68 did not stimulate shoot regeneration from the cotyledon explants where their petiole were removed (King *et al* 1991.)

The growth-stimulating effect of Pluronic F-68 in cultures is of significance because of the economic importance of the genus *Corchorus*. Additionally, manipulations which maximize shoot production will have application in plant genetic engineering. The preliminary experiment on cultured root explants of *Arabidopsis thaliana* with Pluronic F-68 shows that, Pluronic F-68 can enhance shoot regeneration from *Arabidopsis thaliana* (Ribeiro *et al.* 1992).

There are clear variations in the responsiveness of different plant cell and tissue cultures to increasing concentrations of Pluronic F-68 its purified fraction. Culture of jute cotyledons with attached petiole in the presence of purified Pluronic F-68 up to 0.5% (w/v) increased both shoot and callus growth, with no further

stimulation at higher concentrations (Kumar *et al.* 1991, 1992). Furthermore, cell suspentions of *S. dulcamara* grew normally in the presence of Pluronic F-68 up to 0.08% (w/v), but growth was inhibited at higher concentrations (King *et al.* 1990).

2.3 *Agrobacterium*–mediated genetic transformation of jute

2.3.1 Concept of genetic transformation

Genetic transformation of cells by uptake 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 genes are required to be incorporated into the plant cells prior to the integration of gene/ genes of interest. This reporter gene can be recognized n the plant tissue with the help of selectable agents, confirming transformation of he plant tissue (Gardner, 1993).

Tissue culture technology is an important component of biotechnology, used for the genetic manipulation of crop plants. Although these techniques can play an important role in crop improvement, but contribute very little in the production of disease and pest resistant plants, which is crucial now a day. To overcome such problems in crop improvement genetic transformation of crop plants has been evolved, which offers the ability to introduce single new character into a plant cultivar without altering its existing traits (Gardner, 1993). Thus, genetic transformation provides tools to compliment conventional crop improvement program for developing new varieties with desirable traits, which is not possible through breeding and tissue culture alone.

2.3.2 *A. tumefaciens*- as a natural genetic engineer of dicots

Plant transformation mediated by *A. tumefaciens*, a soil dewelling 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.

2.3.3 Biology of *A. tumefaciens*

These compounds produce 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 for *A. tumefaciens and* Ri in the case of *A. rhizogenes*. Ti and Ri plasmids are classified according to the T-DNA, a mobile segment of Ti and Ri plasmid is transferred to the plant cell nucleus and integrated into the plant chromosome. 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 cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contain the genes for opine catabolism produced by the crown gall cells, and region for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regular organized in six operons that are essential for the T-DNA transfer (*vir A, vir B, vir D,* and *vir G)* or for the increasing of transfer efficiency (*vir C* and *vir E*) (Zupan and Zambryski, 1995, Hooykaas and Schilperoort, 1992, Jeon *et al.*, 1998). Different chromosomal-determined genetic elements have shown their functional role in the attachment of

A. tumefaciens to the plant cell and bacterial colonization: the loci *chvB* and *chvA*, involved in the synthesis and excretion of the b-1,2 bacterial chemotaxis (Ankenbaur *et al.*, 1990, Cangelosi *et al.*, 1990, 1991); the *cel* locus, responsible for the synthesis of cellulose fibrils (Matthysse, 1983); the *pscA* (*exoC*) locus plsaying its role in the synthesis of both cyclic glucagons and acid succinoglycan (Cangelosi *et al.*, 1987, 1991).

2.3.4 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 Deblaere *et al.*, 1985; Torisky *et al.*, 1997).

2.3.5 The need for genetic transformation in jute

Genetic variability is limited in both species, more so in *C. capsularis*, which is completely self-pollination. The cultivars of *C. olitorius* show 10% cross-pollinated crop. Since selection has limited scope in bringing about improvement in both species, ionizing radiation and chemical mutagens have been used in the past to produce desirable varieties. However, the use of ionizing radiation did not prove very fruitful. For instant, during the last 30 years or so, only one variety of *C. capsularis* (Atom Pat 38) has been released by the Bangladesh Institute of Nuclear Agriculture through the use of ionizing radiation. (Islam *et al.*, 1992).

2.3.6 Genetic transformation in jute

Foreign genes may be introduced into the nucleus of many dicots by *Agrobacterium* mediated transformation (Klee *et al.*, 1987; Weising *et al.*, 1988). Jute plants can be easily infected by *A. tumefaciens*, because jute is a dicotyledonous plant. Gene was delivered into the cut surface of the meristematic zone of both plumule and cotyledonary bases as was evident by GUS assay. However, they found no mature transgenic plants (Hossain *et al.*, 1998).

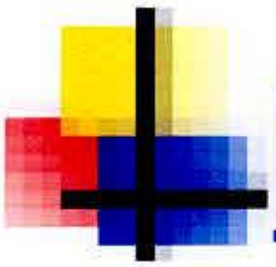
During the last few years, substantial progress has been made in the development of transformation system of crop plants. Successful transfer of genes using *Agrobacterium* as a carrier has been achieved in a number of crops.

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.

Khatun *et al.* (1990) and later in 1993 first reported genetic transformation of jute. Hypocotyls and cotyledons of *C. capsularis* and *C. olitorius* were inoculated with *A. 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 hairy roots from the infected regions. 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

(Khatun *et al.* 1990; 1993). Multiple shoot-buds were regenerated from *Agrobacterium*-infected cotyledonary petioles of *C. capsularis* (Khatun *et al.* 1994).

CHAPTER-3



MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Location, time, duration and year

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

3.2 Experimental Materials

Cotyledons with attached petioles of *C. olitorius* jute plant having the variety O-9897, O-72, O-4 and OM-1 were used in the present investigation.

3.3 Sources of the experimental materials

The seeds of *C. olitorius* used in the experiment were collected from Manikgonj substation of Bangladesh Jute Research Institute (BJRI), Dhaka. The strain of *A. tumefaciens* used in this study was obtained through the courtesy of Biochemistry and Molecular Biology Department, Dhaka University.

3.4 Media used

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

3.4.1 Experiment-A: *In vitro* regeneration potentiality of *C. olitorius* varieties

A. Seed germination

MS (Murashige and Skoog, 1962) basal medium was supported with clinical cotton or agar.

B. Callus induction and shoot differentiation

i. MS medium as control

ii. MS medium supplement with 3.0 mg/l BAP and 0.5 mg/l IAA

C. Root initiation

i. MSO medium (hormone free MS medium).

3.4.2 Experiment-B: *Agrobacterium*-mediated genetic transformation of *C. olitorius* varieties

A. Seed germination

MS basal medium supplemented with clinical cotton.

B. *Agrobacterium* culture and inoculation

Culture media, namely, YMB (Yeast extract Mannitol Broth) medium, was used with kanamycin as antibiotic to grow genetically engineered *A. tumefaciens*. Here two sorts of media were used, such as *Agrobacterium* maintenance medium and *Agrobacterium* working culture medium for transformation.

C. Co-cultivation

MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IAA.

D. Washing of explants after co-cultivation

MS liquid medium supplemented with 500 µg/ml cefotaxime

F. Selection and regeneration

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

3.5 Procedures of media preparation

3.5.1 Preparation of culture media

In this trial, for the induction of callus and plantlet regeneration in jute a number of culture media have been prepared and advocated of which MS medium was used for the present research work. A nutrient medium consists 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 presented in Appendix I.

3.5.2 Preparation of stock solutions

Stock solutions of 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. Separate stock solutions for macronutrients, micronutrients, vitamins and growth regulators were prepared and stored appropriately for use. Iron was directly used as powder.

i) Stock solution A (macronutrients)

The stock solution of macronutrients was made up to 10 folds (10x) 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 (micro-nutrients)

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

iii) 0.028 gm Ferrous sulphate powder was directly added to the medium.

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 75 ml of distilled water. Then the final volume was made up to 100 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots and stored at -20°C. Myo-inositol was used directly as powder at the time of media preparation.

v) Hormonal stock solutions

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) were used in the present investigation.

A. Auxin: 3-indole acetic acid (IAA)

B. 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 in a clean beaker and dissolved in 1 ml of the particular solvent. The mixture was then collected in a 20 ml measuring cylinder and volume was made up to 10 ml by the further addition of distilled water. The solution was then poured into a clean glass container and stored at 4°C and used for maximum period of two weeks.

3.5.3 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:

3.5.4 Preparation of MS medium

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

i) 100 ml of macronutrients, 10 ml of micronutrients, 0.028 gm iron powder and 10 ml of vitamins were taken from each of these stock solutions into a 2 liter Erlenmeyer flask on a heater cum magnetic stirrer.

ii) 100 mg of myo-inositol was added directly to the solution and dissolved.

iii) 30 grams of sucrose was added to this solution and agitated gently to dissolve completely.

iv) Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

v) pH of the medium was adjusted to 5.8 with a digital pH meter with the help of 0.IN NaOH or 0.IN HCl, whichever was necessary.

vi) The whole mixture was then made up to 500 ml .

vii) 7.5 gm agar was added to solidify the medium. The mixture was then heated gently with continuous stirring till complete dissolution of agar. Medium was then mixed thoroughly with dissolved agar (500 ml).

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

3.6 Sterilization

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

3.6.1 Sterilization of culture media

The conical flasks containing prepared media were autoclaved at 1.16 $kgem^{-2}$ pressure and 121^{0}C temperature for 20 minutes. The medium was then poured into sterile Petri dishes and sterile culture vessels (flasks) in a laminar air flow cabinet and were allowed to be cool before use. All the Petri dishes and vials were marked with permanent marker or sticker to indicate specific phytohormone combinations.

3.6.2 Sterilization of glassware and instruments

Beakers, test tubes, conical flasks, pipettes and metallic instruments like forceps, scalpels, inoculation loops micropipette tips, eppendorf tubes, needles, spatulas were wrapped with brown paper packet vials were capped with plastic cap and then were sterilized in an autoclave at a temperature of 121 °C for 20 minutes at 1.16 $kgcm^{-2}$ pressure.

3.6.3 Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all floors and walls with a detergent followed by wiping with savlon. The process of sterilization was repeated at regular intervals. Generally, laminar airflow cabinet was sterilized by wiping the working surface with 95% ethyl alcohol.

3.6.4 Precautions to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar air flow 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.

3.7 Culture techniques

3.7.1 Protocol for *in vitro* seed germination of *C. olitorious*

Seeds of *C. olitorius* (vars. O-9897, O-72, O-4 and OM-1) were surface sterilized by immersion in 0.1% (w/v) Mercuric Chloride for 20 minutes, followed by 6 washes with sterile distilled water. Seeds are germinated on the surface of 50ml aliquots of hormone free agar-solidified (0.8%, w/v) MS basal medium contained in 100 ml capacity of conical flasks. Fifty seeds were inoculated in three flasks.

In another set of experiment, surgical cotton was used instead of agar in association with MS basal medium. Cotton-supported liquic medium was used for seed germination. Surgical cotton (3 mg) was placed at the bottom of 100ml flasks. Each flask contained 25ml of hormone free MS liquid medium. Seeds of *C. olitorius* varieties were surface sterilized with 0.1% (w/v) Mercuric Chloride for 20 min and placed on the surface of cotton-based MS medium. Cultures were placed in a growth room with 28^{0}C under 1.0 Wm^{-2} of daylight fluorescent tubes with 12 hour photoperiod. Fifty seeds were inoculated in three flasks. 8-9 days old seedlings were used for data collection.

3.7.2 *In vitro* callus induction of *C. olitorius*

The following culture methods were employed in the present investigation:

a) Explant culture

b) Subculture

a) Explant culture

The seedlings raised in axenic culture were used as the source of explants. The cotyledons with attached petioles were used as explants. Cotyledons with attached petioles of *C. olitorius* were taken from *in vitro* grown seedlings for this study. Seedlings were allowed to be developed for 8-9 days to make sure that the undeveloped apical shoot buds were not attached with the petioles. Therefore, the optimum explants cotyledons with their attached petioles were excised from 8-9 days old seedlings and were cultured in 250ml conical flasks containing 50 ml of agar-solidified MS medium supplemented with IAA (0.5 mg/l) and BAP (3.0 mg/l). Eight explants were inoculated in each culture flask.

The culture flasks containing explants were placed in a growth room and maintained at 28^{0}C under 1.0 Wm^{-2} of daylight fluorescent tubes with a 12 hrs photoperiod. The culture flasks were checked daily to note the response of contamination. Data were recorded 6 weeks after culture.

b) Subculture

i) Subculture of the callus for shoot regeneration

Two weeks after inoculation of explants, the calli attained convenient size. Then they were removed aseptically from the cultured flask were placed again on freshly prepared sterilized MS medium without hormone. The culture flasks showed signs of contamination were discarded.

ii) Transfer of regenerated shoot-buds for root induction

When the shoots were 2-3 cm in length, they were rescued aseptically from the cultured flasks and were separated from each other and again cultured individually on 250ml conical flask with freshly prepared MSO (hormone free MS medium) medium for root production. The conical flasks containing plantlets were incubated at 28^0 C under a 1.0 Wm^{-2} of daylight fluorescent tubes with a 12 hrs photoperiod. Day to day observations was carried out to note the responses.

3.7.3 Optimization of shoot regeneration in *C. olitorius* at different concentrations of BAP

The following culture techniques were employed in the present study:

a) Explant culture

b) Subculture

a) Explant culture

The seedling raised in axenic culture was used as the source of explants. Here cotyledons (with attached petioles) were used as explants. Eight explants were inoculated in each culture flask containing different treatments of BAP (1 mg/l, 2 mg/l, 3 mg/l and 4 mg/l) and constant IAA (0.5 mg/l). The culture flasks containing explants were placed under fluorescent light in a growth room with controlled temperature (28^0C).

b) Subculture of the callus for shoot regeneration

Demonstrated in section 3.7.2 (b) (i)

3.7.4 Effects of different concentrations of $FeSO_4$ on shoot regeneration in *C. olitorius*

The following culture techniques were employed in the present study:

a) Explant culture

b) Subculture

a) Explant culture

The seedling raised in axenic culture was used as the source of explants. Here coltyledons (with attached petioles) were used as explants. Eight explants were inoculated in each culture flask containing different concentrations of $FeSO_4$ (0 mg/l, 28 mg/l, 56 mg/l, 84 mg/l and 112 mg/l). The culture flasks containing explants were placed under fluorescent light in a growth room with controlled temperature (28^0C).

b) Subculture of the callus for shoot regeneration

Demonstrated in section 3.7.2 (b) (i)

3.7.5 Study of surfactants (Pluronic F-68)

The experiment was undertaken to investigate the effects of Pluronic F-68 on shoot regeneration from cotyledons with attached petiole of *C. olitorius*. Determination of a possible relationship between the physico-chemical properties these individual compounds and their observable effects on plant morphogenesis in cultured jute cotyledons was also studied (Kumar *et al* 1992).

3.7.5.1 Culture of jute tissue with Pluronic F-68

The objectives of these experiments were firstly, to determine the effect of Pluronic F-68 on increasing the frequency of shoot regeneration. Secondly, to observe any apparent harmful effect on regenerated matured plants.

For the first set of works, cotyledons with attached petiols of *C. olitorius* (vars. O-9897, O-72) were excised from 7 d old seedlings and cultured in 60 z capacity jars (6 cotyledons/jar) on the surface of 50 ml aliquots of agar solidified MS-based medium containing the plant growth regulators, IAA (1.0 mg/l) and BAP (3.0 mg/l) for control. For experimental purposes, the growth medium was supplemented with 0.005, 0.01, 0.02, 0.04, 0.08% (w/v) of purified fraction of Pluronic F-68. Excised cotyledons were also cultured on MSO agar solidified medium containing Pluronic F-68 at the same concentrations for control. Cultures were incubated at 25^{0}C with 16 h (0.5 Wm^{-2}) of daylight fluorescent illumination). The number of shoots produced per explant were recorded after 7 weeks of culture. Each experiment was repeated 3 times with 3 replications.

In the second set of the experiments, the cotyledons of *C. olitorius* (vars. O-9897 and O-72) were cultured similarly on MS-based medium containing IAA (1.0 mg/l) and BAP (3.0 mg/l), with or without Pluronic F-68 as described above. As IAA with BAP produced plants with vitrified leaves, the emerging shoots needed to be transferred onto MS-based medium where IAA and BAP were replaced with zeatin (1.0 mg/l), after 3 weeks of culture initiation. The aim of this experiment was to determine whether Pluronic F-68 has any harmful effect on the developing plantlets. After 3 weeks, these developing shoots (1-2 cm in length) were transferred to agar-solidified MSO medium for rooting. Plants (2-3cm in height) regenerated in the presence or absence of Pluronic F-68, were potted in 9 cm diameter plastic pots, transferred to the glasshouse and grown to maturity .

3.8 *Agrobacterium* strain and plasmid

Genetically engineered *A. 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).

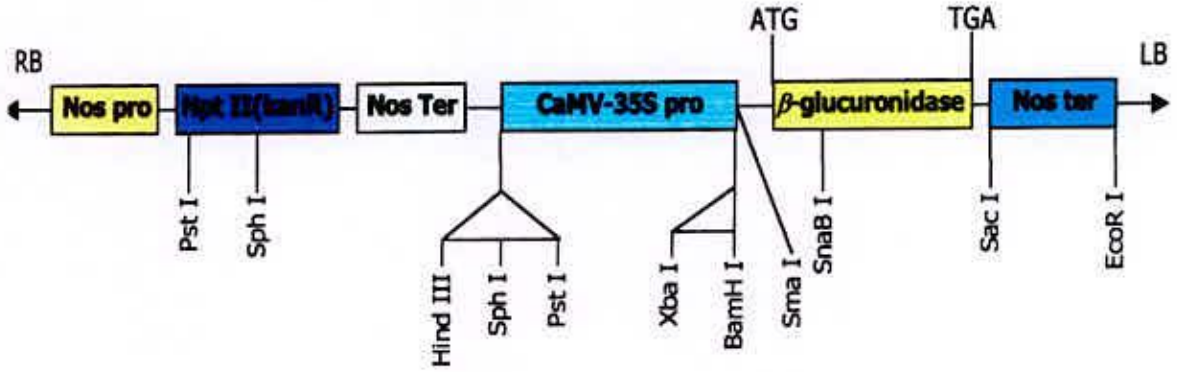


Fig 1. pBI121 –Region between left border (LB) and right border (RB)

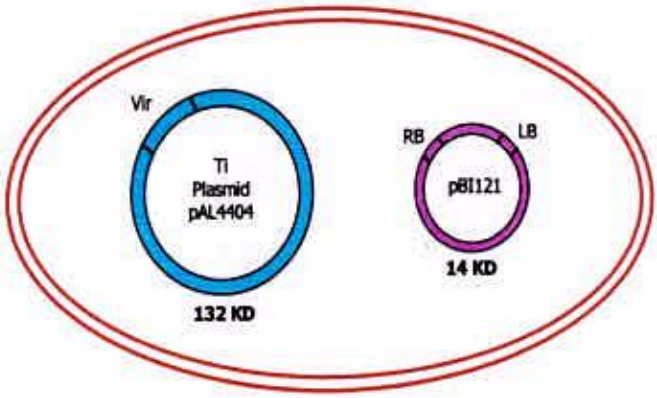


Fig 2. *Agrobacterium tumefaciens* LBA4404

3.8.1 Preparation of *Agrobacterium* culture medium

3.8.1.1 Preparation of YMB (Yeast Extract 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:

Mannitol	1 %
Yeast Extract	0.04%
$MgSO_4.7H20$	0.02%
NaCI	0.01%
KH_2PO_4	0.05%

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

For preparing stock solution of kanamycin, 0.5 g of this antibiotic was dissolved in 20 ml of distilled water. It was then filter sterilized and stored in eppendorf tubes at -20°C in dark as stock.

3.8.1.2 Preparation of GUS assay solution:

Various β-glucuronic acid substrates are available for detection of GUS expression *in vitro*. All of these substrates contain the sugar D-glucopyranosiduronic acid attached by glusidic linkage to a hydroxyl group of a chromogenic, fluorogenic, or other detectable molecule. The preferred substrate for GUS detection in 5-bromo-4chloro-3-indolyl- β-D-glucuronide or X-gluc. 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). However, GUS staining solution is composed of following chemicals with there concentration.

Components	Amount/5ml
X-gluc (solvent : DMSO)	4.445mg
Chloramphenicol	100µl
NaH_2PO_4	59.9mg
Triton (10%) X	50ul
Methanol	1ml

X-gluc stock: 10mg X-gluc in 1ml DMSO.

3.8.1.3 Experiment-B *Agrobacterium*-mediated genetic transformation of *C. olitorius* varieties

The following culture techniques were employed in the present investigation:

i. Axenic culture

ii. Explant preparation

iii. *Agrobacterium* culture

iv. Infection and incubation

v. Co-cultivation

vi. GUS (β-glucuronidase) histochemical assay

i) Axenic culture

Sterilized seeds were placed onto seed germination medium in 100ml conical flask. In each flask 25-30 seeds were inoculated. The culture was then incubated in incubation room till the germination of seeds. Seven to nine days old seedlings were used as source of contamination-free explants.

ii) Explant preparation

The germinated seedlings raised in axenic culture were used as the source of explants. Cotyledonary petioles were used as explants. After seven days, cotyledons were excised from the seedlings. This was carried out by gently holding the hypocotyls with forceps, and cutting between the joint just below the shoot tip using sterilized surgical blades.

iii) *Agrobacterium* culture

As has been mentioned earlier two kinds of culture media were needed for the *Agrobacterium* strain. One for maintaining *Agrobacterium* stock and the other for the infection of explants. For maintenance, one single colony from previously maintained *Agrobacterium* stocks was streaked into freshly prepared petridish containing YMB medium having kanamycin. The Petri dish was sealed with parafilm and kept in room temperature for at least 48 hours. This was then kept at 4°C to check over growth. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The cultures were subcultured regularly at each week in freshly prepared media to maintain the stock.

For infection, from this *Agrobacterium* stock single streak was taken in an inoculation loop and was inoculated in a conical flask containing liquid LB 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.

iv) Infection and incubation

The *Agrobacterium* grown in liquid LB media were used for infection and incubation. Prior to this "Optical Density" of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer. Following the determination of density, to get suitable and sufficient infection of the explants, freshly excised explants were dipped into bacterial suspension (OD_{600}=0.6) for 1 minutes before transferring them to co-cultivation medium.

v) Co-cultivation

Following infection and incubation, the explants were co-cultured on co-cultivation medium. Prior to transfer of all explants to co-cultivation media they were blotted dry with sterile filter papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-cultivation media for 1-4 days. Co-cultured Petridishes containing explants were placed under fluorescent illumination with 16/8 hours light/dark cycle at 28^0C. The intensity of light was maintained at 1500 lux. The culture vessels were checked daily to discard the contaminated vessels and to note the behavior of the explants.

vi) 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 assays. Immediately after inoculation on selection medium, cotyledons were incubated in GUS staining solution at 37^0C 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.

3.9.1 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 of seed germination

The germination percentage was estimated as ratio of the number of seed germinated to the number of seed 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) Percent of callus induction

Percent callus induction was calculated on the basis of the number of explant placed and the number of callus induced.

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

c) Percent of 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) Days of callus initiation

Generally, callus initiation started after six days of incubation of explants. The number of callus initiation over a number of days was recorded. The mean value of the data provided the days required for callus initiation.

e) Days to shoot initiation

Shoot initiation started after 15-21 days of incubation of explants. The number of shoots proliferated over a number of days were recorded. The mean value of data provided the days required for shoot initiation.

f) Average number of shoot per callus

Some calli produced only single shoot while some produced multiple shoots. So, number of shoot per callus was recorded at 21 days interval and the mean was calculated using the following formula:

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

where,

$\overline{X}$ = mean of shoots/callus

$\sum$ = summation

Xi = number of shoots/callus

n = number of observation

3.9.2 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 means were compared by Duncan's Multiple Range Test (DMRT).

CHAPTER-4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1 Experiment A. *In vitro* regeneration potentiality of *C. olitorius*

4.1.1 *In vitro* seed germination potentiality of *C. olitorius*

The experiment was conducted for the regeneration of healthy seedlings from jute seeds required for subsequent experiments. In the present experiment, seeds of *C. olitorius* (vars. O-9897, O-72, OM-1 and O-4) were germinated on both cotton-supported liquid and agar solidified medium. The number of germinated seeds were presented in percent and its interpretations have been given under the following headings.

4.1.1 Effect of different varieties

Number of seed germination

Significant variations were recorded among the different varieties of *C. olitorius* where 15 seeds were blotted for germination (Table 1). The highest number of germinated seeds (13.00) were counted for the var. O-9897 which was closely followed by the var. O-72 (12.50). On the other hand, the lowest number of germinated seeds (10.50) were counted in the var. O-4. Variation of seed germination occurred due of variation in dormancy in the varieties.

Percent of seed germination

A statistically significant variation was recorded among the different varieties of *C. olitorius* considering the percentage of germinated seeds under the present trial (Table 1). The highest percentage of germination (86.67%) were counted for the var. O-9897 which was closely followed by the vars. O-72 (83.33%). On the contrary, the lowest percentage of germination (61.17%) were counted in the var. O-4. Such results may be due to seeds dormancy or may be physiological reason of different varieties.

Table 1. Seed germination of different varieties of *C. olitorius*

Varieties	No. of germinated seeds /flask*	Percent of seeds germination
O-9897	13.00 a	86.67 a
O-72	12.50 a	83.33 a
OM-1	11.00 b	73.33 b
O-4	10.50 b	70.00 b
$LSD_{(0.05)}$	1.250	8.333
CV	6.65	6.65

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

*Each flask contain 15 seeds.

4.1.2 Combined effects of the varieties and media

Number of seed germination

A significant variation was recorded in reflection of the number of germinated seeds in MS media and the results are presented in (Table 2). The highest number of germinated seeds (13.50) for the var. O-9897 were counted in cotton-supported liquid medium and the lowest germinated seeds (9.50) for the var. O-72 was recorded in agar solidified medium. From these results it was found that cotton-supported liquid media was found the more suitable for the germination of *C. olitorius* than agar solidified medium(Plate 1, Plate 2). Similar results were also reported earlier by Khatun (2003). This might be due to the more aeration facilities of cotton supported liquid media.

Percent of seed germination

The combined effects between the varieties and MS media also showed statistically significant differences in the percentage of seeds germination. The highest percentage of seed germination (90.00%) were recorded in the var. O-9897 in cotton-supported liquid medium (Table 2). The lowest percentage of seed germination (63.33%) were recorded in the var. O-72 with agar solidified medium.

Seeds of the varieties of *C. olitorius* germinated on both agar and cotton-supported MS medium. The varieties ensured 90.00% to 86.67 % seed germination on cotton supported MS liquid medium, whereas, for agar solidified Ms medium, seed germination were as low as 63.33% to 68.33% for the same variety. The differences in percentage of germinated seed between agar supported medium and cotton- supported liquid MS medium were found to be statistically significant.

Table 2. Combined effects of varieties and culture media on seed germination in jute

Varieties × Culture Media		No. of germinated seeds /flask*	Percent of seeds germination
O-9897	Cotton	13.50 a	90.00 a
	Agar	10.25 b	68.33 b
O-72	Cotton	13.00 a	86.67 a
	Agar	9.500 b	63.33 b
	$LSD_{(0.05)}$	0.9327	6.221
	CV	5.05	5.04

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

*Each flask contain 15 seeds.

Plate 1. Seed germination on clinical cotton-supported MS liquid medium

Plate 2. Seed germination on agar-supported MS medium

Similar findings were reported for the seed germination of white jute varieties (Naher *et al.*, 2003). Though the result of seed germination of *C. olitorius* varieties was found to be more marked than *C. capsularis* both of the species responded better in cotton-supported MS liquid medium. The seedlings of *C. olitorius* varieties grown on cotton-supported MS liquid medium were found to be much more healthier than the seedlings grown on agar supported MS medium. Similar response was reported for *C. capsularis*. (Naher *et al.*, 2003). This result could be a valuable addition for tissue culture system as cotton-supported seed germination system was comparatively cheaper than agar supported system. Moreover, medium consumption is lesser (i.e.20ml/flask) for cotton-supported system, whereas, for agar supported system, medium requirement was 50 ml/flask.

4.2.1 Optimization of shoot regeneration in *C. olitorius* at different BAP concentrations

Different concentrations of BAP and constant IAA were used for the shoot regeneration using cotyledonary petioles as explants to know optimum BAP concentrations for plant regeneration.

4.2.2 Combined effects of the varieties and BAP concentrations

Average number of regenerated cotyledons

The combined effects between the varieties and BAP concentrations also showed statistically significant differences in respect of average number of regenerated cotyledon under the present piece of experiment (Table 3). The highest average number of regenerated cotyledons (4.33) were recorded in the var. O-9897 with 3 mg/l BAP which was statistically identical with the var. O-72 (3.66) with 3 mg/l BAP (Plate 3). The lowest average number of regenerated cotyledons (1.667) were counted in the both vars. O-9897 and O-72 with 1 mg/l BAP.

Table 3. Percent of shoot regeneration of different varieties of *C. olitorius* at various concentrations of BAP

Variety × BAP concentrations (mg/l)		No. of shoot producing cotyledons/flask*	Percent of shoot producing cotyledons	Average number of produced shoots by each cotyledon
O-9897	0	0.000 f	0.000 f	0.000 e
	1	1.667 e	27.78 e	7.167cd
	2	3.667 ab	61.11 ab	8.973 b
	3	4.333 a	72.22 a	11.48 a
	4	2.667 cd	44.44 cd	7.943 bc
O72	0	0.000 f	0.000 f	0.000 e
	1	1.667 e	27.78 e	5.833 d
	2	3.333 bc	55.56 bc	8.693 bc
	3	3.667 ab	61.11 ab	10.58 a
	4	2.333 de	38.89 de	7.557 bc
$LSD_{(0.05)}$		0.7298	12.18	1.462
CV		18.26	18.26	12.48

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

*Each flask contain 6 cotyledons.

Plate 3. Shoot regeneration from the vars. O-9897 and O-72 on MS+0.5 mg/l IAA + 3 mg/l BAP (A: O-9897 and B: O-72)

Percent of shoot producing cotyledons

The combined effects between the varieties and concentrations of BAP also demonstrated statistically significant differences in respect of percentage of shoot producing cotyledons under the present trial. The highest percentage of shoot producing cotyledons (72.22%) were recorded in the var. O-9897 with 3 mg/l BAP which was statistically similar with the var. O-72 (61.11) with 3 mg/l BAP (Table3). The lowest percentage of shoot producing cotyledons (27.78%) were recorded in the both vars. O-9897 and O-72 with 1 mg/l BAP.

Average number of produced shoots by each cotyledon

Combined effects between the varieties and BAP concentrations also showed statistically significant differences in respect of average number of produced shoots by each cotyledon under the present trial in laboratory condition. The highest average number of produced shoots by each cotyledon (11.48) were recorded in the var. O-9897 with 3 mg/l BAP which was statistically matching with the var. O-72 (10.58) with 3 mg/l BAP (Table 3). The lowest average number of produced shoots (5.833) by each cotyledon were recorded in the variety O-72 with 1 mg/l BAP.

In this study, differences were noticed among the varieties of *C. olitorius* in percentage of shoot producing cotyledons and also in number of produced shoots by each cotyledon. Similar observation was reported earlier by Khatun *et al.* (1993) and Naher *et .al.* (2003) for plant regeneration from 10 accessions of *C. capsularis* cotyledons. Naher *et. al.* (2003) demonstrated that the differences were noticed among the 10 accessions of *C. capsularis* in percentage of shoot producing cotyledons and also in number of produced shoots by each cotyledon. All these reports were in agreement with the results of the present investigation on jute for varietals differences.

Plant regeneration from different explants of *C. olitorius* and *C. capsularis* have been reported by Khatun (1993). A detail study of cotyledon segments, hypocotyls

segments and root segments have been conducted for morphogenic responses of jute by using various combinations and concentrations of auxins and cytokinins. Plant regeneration was not obtained from any explants including the cotyledons without petioles attached. The present finding also indicates that the cotyledons of *C. olitorius* will respond to plant regeneration provided the petioles remain attached. *Brassica* spp. (sharma *et al.*, 1991); white jute *C. capsularis* (Khatun *et al.* 1993) and kenaf (Khatun *et. al.* 2003) reported these species similarly required an attached petioles for their cotyledons to undergo morphogenesis. Like wise, cultured leaves of *Echeveria elegans* require an attached petiole for plant regeneration (Raju and Mann, 1970).

4.3.1 Effects of different concentrations of $FeSO_4$ on shoot regeneration in *C. olitorius*

To identify the optimum concentrations of $FeSO_4$ for the regeneration of *C. olitorius* the cotyledons were used as explants in this work.

4.3.2 Combined effects of varieties and different concentrations of $FeSO_4$ Number of shoot producing explants

The combined effects of varieties and concentrations of $FeSO_4$ also showed a statistically significant difference in respect of number of shoot producing explants under the present piece of experiment. The highest number of shoot producing cotyledons (4.667) were recorded in the var. O-9897 at 56 mg/l of $FeSO_4$ which was statistically identical (4.333) for the var. O-72 at 56 mg/l of $FeSO_4$ (Table 4). The lowest number of shoot producing cotyledons (2.333) were recorded in the both vars. O-9897 and O-72 at 112 mg/l of $FeSO_4$.

Percent of shoot regeneration

The combined effects of varieties and different concentrations of $FeSO_4$ also showed a statistically significant difference in respect of percent shoot regeneration. The highest percent of shoot regeneration (77.78%) were recorded in the var. O-9897 at 56 mg/l of $FeSO_4$ which was statistically identical (72.22%) with the var. O-72 at 56 mg/l of $FeSO_4$. The lowest percent of shoot regeneration (38.89%) were counted for the both vars. O-72 and O-9897 at 112 mg/l of $FeSO_4$. (Table 4; Plate 4).

Average number of produced shoots by each cotyledon

Combined effects between the varieties and $FeSO_4$ concentrations also showed statistically significant differences in respect of average number of produced shoots by each cotyledon under the present trial in laboratory condition. The highest average number of produced shoots (11.43) by each cotyledon were recorded in the var. O-9897 at 56 mg/l of $FeSO_4$ which was statistically matching (10.90) with the var. O-72 at 56 mg/l of $FeSO_4$ (Table 4). The lowest average number of produced shoots (8.057) by each cotyledon were recorded in the variety O-72 at 112 mg/l of $FeSO_4$.

Table 4. Combined effects of different varieties of *C. olitorius* and different concentrations of $FeSO_4$ on shoot regeneration

Varieties × Concentrations of $FeSO_4$ (mg/l)		No. of shoot producing cotyledons/flask*	Percent of shoot producing cotyledons	Average no. of produced shoots by each cotyledon
O-9897	0	0.000 f	0.000 f	0.000 f
	28	3.667 bc	61.11 bc	10.31 bc
	56	4.667 a	77.78 a	11.43 a
	84	2.667 de	44.44 de	9.833 cd
	112	2.333 e	38.89 e	8.110 e
O-72	0	0.000 f	0.000 f	0.000 f
	28	3.333 cd	55.56 cd	10.81 ab
	56	4.333 ab	72.22 ab	10.90 ab
	84	2.667 de	44.44 de	9.223 d
	112	2.333 e	38.89 e	8.057 e
$LSD_{(0.05)}$		0.6688	11.14	0.8979
CV		14.99	14.99	6.66

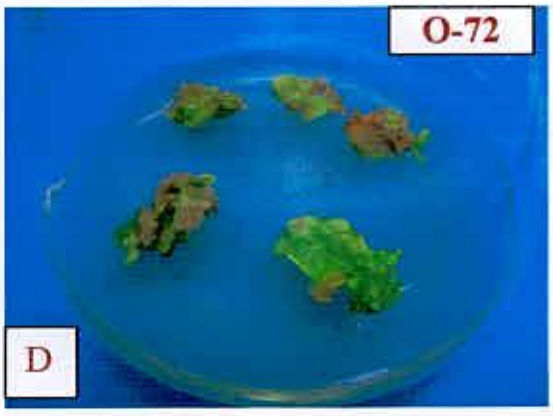
Figures followed same letter in a column do not differ significantly by DMRT.

*Each flask contain 6 cotyledons.

A. Shoot regeneration from the cotyledons of *C. olitorius* (O-9897) at 56 mg/l of $FeSO_4$

B. Shoot regeneration from cotyledons of *C. olitorius* (O-9897) at 112 mg/l of $FeSO_4$

C. Shoot regeneration from cotyledons of *C. olitorius* (O-72) at 56 mg/l of $FeSO_4$



D. Shoot regeneration from cotyledons of *C. olitorius* (O-72) at 112 mg/l

Plate 4. Comparisons of shoot regeneration of O-9897 and O-72 on MS media from cotyledons of *C. olitorius* at different concentrations of $FeSO_4$

4.4.1 Influence of Pluronic F-68 on *C. olitorius* explants

Number of shoot producing cotyledons

Shoot regeneration was not stimulated by the presence of Pluronic F-68 from the cotyledons without petiole from any of the species. Only callus was produced. Such callus grew at all concentrations and combinations of growth regulators tested. In the presence of IAA (1.0 mg/l) with BAP (3.0 mg/l), maximum shoot producing cotyledons (4.333) were counted for the var. O-9897 and 4.00 for the var. O-72 (table 5) at 0.08% Pluronic F-68 (Plate 5).

4.4.2 Effects of Pluronic F-68 on shoot regeneration

Supplementation of culture medium with 0.005-0.08% (w/v) Pluronic F-68 markedly enhanced the percentage of shoot producing cotyledons and stimulated the number of shoots production per cotyledon (Table 5; Plate 4). The hieghest percentage of shoot producing cotyledons (72.22) were in the var. O-9897 which was closely followed by the var. O-72 (66.67), the maximum number of shoots (11.55) per cotyledon were in the presence of 0.08% Pluronic F-68 (Plate 4 A) which was closely followed by the var. O-72 (11.25). The stimulation of shoot regeneration from cultured cotyledons with attached petiole by purified Pluronic F-68 was also reflected in the developing shoots and regenerating shoots.

Table 5. No. of regenerated explants and shoots per explant on different varieties of *C. olitorius* in presence of growth hormone (IAA & BAP) supplemented with purified Pluronic F-68

Varieties	Concentrations	Purified		
	Pluronic F- 68 (w/v, %)	No. of shoot producing cotyledons/flask*	Percentage of shoot producing cotyledons	Average No. of produced shoots/ cotyledon
O-9897	0.0	3.333 b	55.56 b	6.443 f
	0.005	3.667 ab	61.11 ab	6.333 f
	0.01	3.333 b	55.56 b	8.193 e
	0.02	3.333 b	55.56 b	9.390 d
	0.04	3.667 ab	61.11 ab	10.36 c
	0.08	4.333 a	72.22 a	11.55 a
O-72	0.0	3.333 b	55.56 b	6.363 f
	0.005	3.333 b	55.56 b	6.360 f
	0.01	3.333 b	61.11 ab	7.807 e
	0.02	3.667 ab	61.11 ab	9.193 d
	0.04	3.667 ab	61.11 ab	10.47 bc
	0.08	4.000 ab	66.67 ab	11.25 ab
$LSD_{(0.05)}$		0.8330	12.92	0.8209
CV		13.74	12.68	5.61

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

*Each flask contain 6 cotyledons.

Plate 5. Influence of Pluronic F-68 on shoot regeneration from the petiole of excised cotyledons of *C. olitorius* (vars. A: O-9897 and B: O-72).

4.5 Experiment-B. *Agrobacterium*- mediated genetic transformation of *C. olitorius* genotype

Genetic transformation is a powerful and important tool which can be used in jute improvement program. But an efficient and reproducible transformation protocol is required for successful genetic transformation. Therefore, in the present study, investigations were made to generate transgenic plant from two varieties of *C. olitorius* (vars. O-9897 and O-72) through *Agrobacterium*-mediated transformation using cotyledons (with attached petiole) as explants.

4.5.1 Combined effects of varieties and *A. tumefaciens*

Number of shoot producing cotyledons

The combined effects of varieties and *A. tumefaciens* also showed a statistically significant difference in respect of number of explants produced shoot under the present piece of experiment. The highest number of shoot producing cotyledons (3.60) were recorded in the var. O-9897 with *A. tumefaciens* which was statistically identical (3.50) with the var. O-72 (Table 6).

Percent of Shoot regeneration

The combined effects of variety and *A. tumifaciens* also showed a statistically significant difference in respect of percent shoot regeneration under the present piece of experiment. The highest percent of shoot producing cotyledons (60.00%) were recorded in the var. O-9897 with *A. tumefaciens* which was statistically identical (58.33%) with the var. O-72 (Table 6).

Average number of produced shoots by each cotyledon

The average number of produced shoots by each cotyledon under the present trial in laboratory condition. The highest average number of shoots (8.883) produced by each cotyledon were recorded in the var. O-9897 which was statistically matching

with the var. O-72 (8.563) (Table 6). The lowest average number of produced shoots (8.057) by each cotyledon were recorded in the variety O-72.

4.5.2 Histochemical GUS (β-glucuronidase) assay

After infection of the explants in *Agrobacterium* suspension culture, the explants were transferred 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 20% inoculated explants tissue. In the GUS assay, conspicuous GUS positive (blue colour) region were detected in the explant surface (Plate 6).

Table 6. Effects of variety towards *A. tumefaciens*

Varieties	No. of shoot producing cotyledons	Percent of shoot producing cotyledons	Average no. of shoots/cotyledon
O-9897	3.600 a	60.00 a	8.883 a
O-72	3.500 a	58.33 a	8.563 b
$LSD_{(0.05)}$	0.5276	8.799	0.1319
CV	14.70	14.70	1.48

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

*Each flask contain 6 cotyledons.

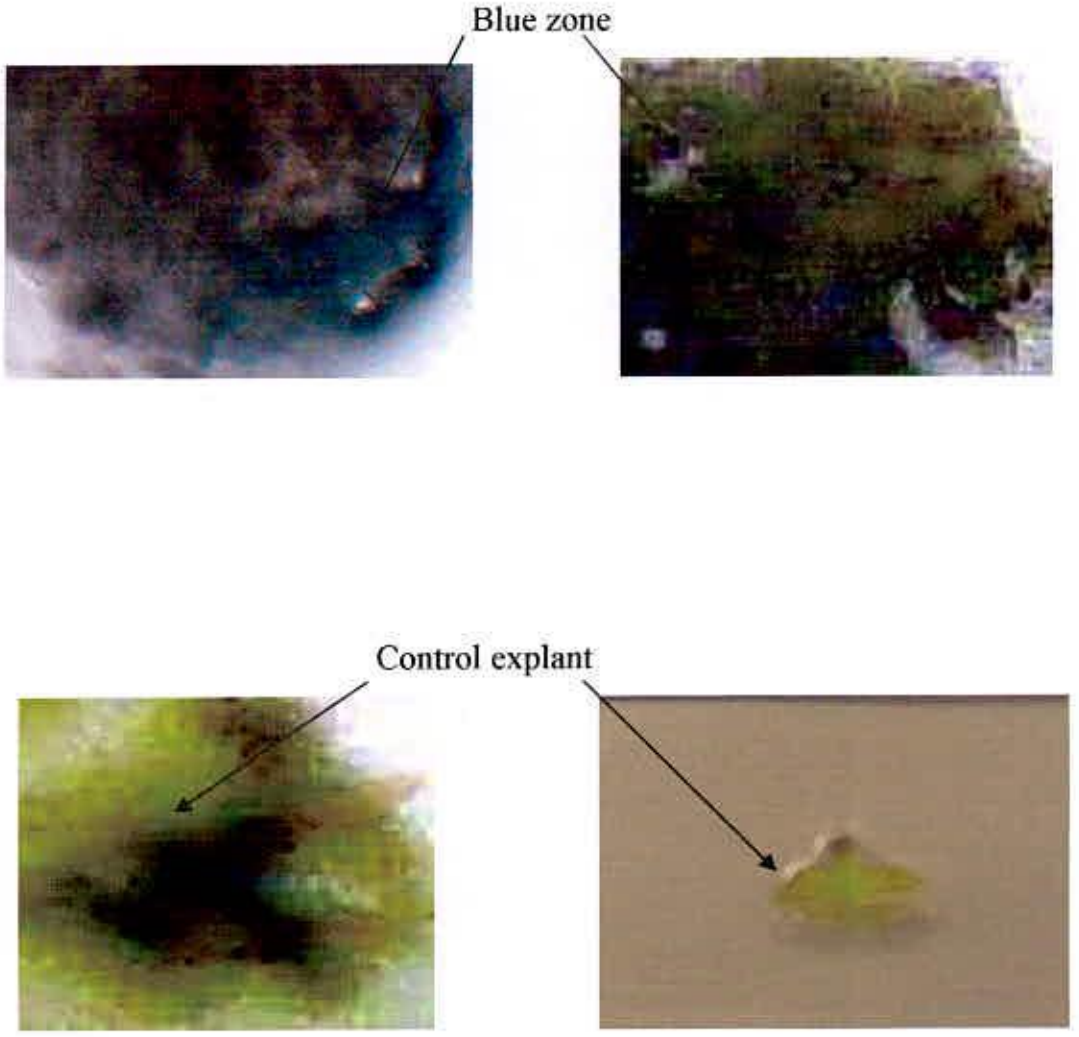


Plate 6. Histochemical localization of GUS activity (blue zone) at the infected callus and cotyledonal tissue (upper) with control explant (lower).

4.5.3 Effects of vaireties

Following GUS histochemical assay, it was found that the two varieties showed positive responses towards transformation.Between the vars. O-9897 showed the highest response (86.67%) to GUS assay and O-72 showed the lowest response (80.00%) to GUS assay (Table. 7). Control explants did not show any response to GUS assay.

4.5.4 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 influenced by the emergence of transgenic shoot from the transformed callus. A few of the calli continued to grow and differentiated into shoots (Plate 7). However, most of the calli failed to produce shoot and died in course of time.

Table 7. Effect of variety towards GUS histochemical assay.

Varieties	No. of infected cotyledons	No. of assayed cotyledons for GUS	No. of cotyledons +ve for GUS	Percent of GUS +ve cotyledons
O-9897	60	15	13	86.67
O-72	60	15	12	80.00

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

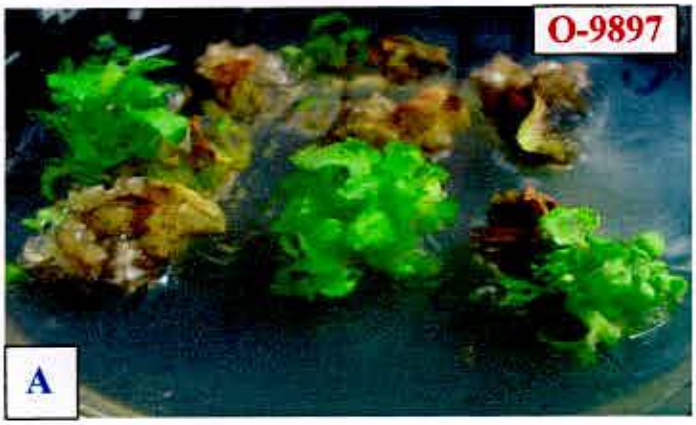
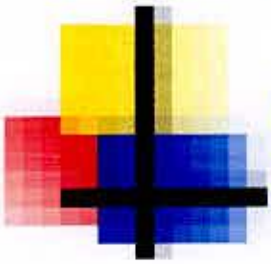


Plate 7. Putative transgenic shoots on selection media (kanamycin and cefotaxime treated) from the vars. O-9897 (A) and O-72(B)

CHAPTER-5



SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

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

In the first set of the experiments, a detailed investigation was carried out to study the seed germination and subsequent plant regeneration of *C. olitorius* genotypes using cotyledons (with attached petioles) as explant. In the second set of the experiments, investigation was carried out to study the *Agrobacterium*-mediated genetic transformation of *C. olitorius*.

Significant variations were reported between the varieties of *C. olitorius* for germination percentage. The highest germination percentage (86.67%) were recorded for the var. O-9897 and the lowest germination percentage (70.00%) were recorded for the var. O-4. In consideration of media composition, cotton-supported liquid media showed better performance (90.00% in O-9897 and 86.67% in O-72) in respect of seed germination.

The highest average number of regenerated cotyledons (4.33) were recorded for the var. O-9897 in the presence of 3 mg/l BAP and the lowest number of regenerated cotyledons (1.667) were recorded in the both vars. O-9897 and O-72 with 1 mg/l BAP. Considering the average number of regenerated cotyledons were significant variation at different BAP concentrations. The highest number of regenerated cotyledons (4.333) were recorded in the presence of 3 mg/l BAP and the lowest number of regenerated cotyledons (1.667) were recorded with 1 mg/l BAP.

Significant variations were recorded between the varieties of *C. olitorius* for the percent of shoot regeneration. The highest percent of shoot producing cotyledons (72.22%) were recorded for the var. O-9897 with 3 mg/l BAP.

Significant variations were recorded in considering the number of produced shoots from explants at different concentrations of $FeSO_4$. The highest number of shoot producing cotyledos (4.667) were recorded at 56 mg/l of $FeSO_4$ and the lowest number of shoot producing cotyledons (2.333) were recorded at 112 mg/l of $FeSO_4$. The variety O-9897 showed better performance in the same $FeSO_4$ concentration.

Considering the percent shoot regeneration significant variations were found in different conc. of Pluronic F-68. The percentage of shoot regeneration was increased up to 0.08% of surfactants and the percent shoot regeneration was decreased in absence of surfactants (0.0%).

In the present study, it was observe that clinical cotton-supported MS liquid media showed high performance in seed germination. Different combinations and concentrations of BAP and constant IAA were used to observe shoot regeneration. The highest percent of shoot regeneration (72.22%) were found in the combination of MS+3 mg/l BAP + 0.5 mg/l IAA. No callus induction and shoot regeneration were found without BAP and IAA. It was worth noting that percent shoot regeneration gradually increased with the increasing of BAP level upto 3 mg/l. Further increasing of BAP level did not show any improvement to shoot formation.

Histochemical GUS assay was performed soon after co-cultivation of explants with the bacterium. *A. tumefaciens* strain LBA4404 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 varieties. Between the vars. O-9897 produced the highest response (86.67% GUS positive) to GUS assay and O-72 produced the lowest response (80.00% GUS positive) to GUS assay.

Following infection and co-cultivation, the explants were cultured on selection and plant regeneration media containing kanamycin along with cefotaxime and growth regulators. *A. tumefaciens* strain LBA4404 has *nptII* gene within its T-DNA, which confers kanamycin resistance of transformed cells. The putative transformed regenerated plantlets grew on selection medium but died in course of time.

In the first set of the experiments, an efficient and reproducible protocol for the regeneration of *C. olitorius* 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 *C. olitorius*.

An efficient protocol for the transformation of *C. olitorius* varieties has been developed in the second set of the experiments, which showed the integration of two marker genes (GUS and *nptII*). Thus, in the future program agronomically important gene/genes can be transferred to *C. olitorius* genotypes using this protocol. Particularly, for the development of disease and insect resistance in *C. olitorius* varieties, this technique of transformation can be exploited successfully.

REFERENCES

REFERENCES

Agarwal, V. K. (2000). Breeding tehnique of jute, Kenaf and mesta. Consultency Report, ARMR (Winrock international), BARC, Dhaka. pp. 56-57.

Ahmed, G., Hossain, A. B. M. M. and Islam, M. S. (1999). Regeneration of multiple shoots in jute *C. olitorius* (var. O-4) from cotyledon and hypocotyls explants of germinating seeds. *Ind. J. Exp. Bio.* **27**: 334 -337.

Ahmed, T. U., Jalil, A. F. M. and Abdul, M. (1993). Bangladesher Krishir Onistakari Pokamakar: Ziban Brittanta O Nyantran. Life history and control of insects harmful to Bangladesh agriculture. BARI Vol- **I** : pp. 131-135.

Akter, N. (2001). Effects of different explants and concentration of NAA on callus induction and plant regeneration of brinjal cv. Uttara. M. Sc. Thesis, BAU, Mymensingh, Bangladesh. pp. 255-257.

Ali, M. A. (1992). *In vitro* studies in *Corchorus* and *Ruphunu.s.* Ph. D. Thesis, Reading University, UK. pp. 66-68.

Ankenbauer, R. G. and Nester, E. W. (1990). Sugar-mediated induction of *A. tumefaciens* virulence genes: structural specificity and activities of monosaccharides. *J. Bacteriology* **172**: 6442-6446.

Bajaj, J. (1989). Genetic manipulation of fibrous plant. Ph. D. Thesis, IARI, New Delhi, India. pp. 344-345.

BBS. (2005). Statistical Pocket Book of Bangladesh. Bangladesh Bureau of Statistics. Planning Division, Ministry of Planning. Govt. Peoples Republic of Bangladesh. pp. 155-158.

Bentley, P. K., Davis, S. S., Johnson, O. L., Lowe, K. C., and Washington, C. (1989). Purification of Pluronic f-68 for perfluorochemical emulsification. *J. Pharm. Pharmacology*. **41**: 661-663.

Bigaria, A. K. (1988). Influence of indole-3-butyric acid, environmental factors and posture on the regeneration of stem cuttings and leaves of *Hibisiscus cannabinus. Advances in Plant Sciences*. **22**(1): 54-66.

BJRI. Annual Report. (2003-2004). Bangladesh Jute Research Institute, Manik Mia Avenue, Dhaka. pp. 11-13.

Carlson, P. S. (1975). Crop improvement through techniques of plant cells and tissue cultures. *J. Biol. Sci.* **25**: 747-749.

Cangelosi, G. A., Ankenbauer, R. G., and Nester, E. W. (1990). Sugars induce *the A. virulence* genes through a periplasmic binding protein and a transmembrane signal protein. *Proceedings of the National Academy of Sciences USA*. **87**: 6708-6712.

Cangelosi, G.A., Best, E. A., Martinetti, C. and Nester, E. W. (1991). Genetic analysis *of A. tumefaciens. Methods in Enzymology* **145**: 177-181.

Cangelosi, G. A., Hung, L., Puvanesarajah, V., Stacey, G., Ozga. D. A., Leigh, J. A. and Nester, E. W. (1987). Common loci for *A. tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their role in plant interaction. *J. Bacteriology*. **169**: 2086-2091.

D'Aamato, F. (1978). Chromosome number variation in cultured cells regenerated plants. **In**: T. A. Thorpe (ed). Frontiers of plant Tissue Culture. Canada. pp. 247-295.

De Block, M. (1988). Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *A. tumefaciens*. *Theor. Appl. Genet.* **76**: 767-774.

Deblaere, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M. and Leemans, J. (1985). Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucleic Acid Research* **13**: 4777-4788.

Debnath, S. C., Mc Cabe, M., Curtis, I. S., De Laat, A., Power, J.B. and Davey, M.R. (1995). *Agrobacterium*- mediated transformation of lettuce cotyledons and regeneration of transgenic plants. Abstracts 2nd Intl. Plant Tissue Culture Conference. December 10-12, Dhaka. p. 32.

Dodds, J. H. and Roberts, L. W. (1990). Anther and Pollen culture. **In**: J. Experiments in Plant Tissue Culture..Dodds and L. W. Roberts, (ed.). Cambrige University Press, New York. pp. 157-171.

Fillatti, J. J., Sellmer, J., Mc Cown, b., Hassing, B., and Comari, L. (1987). *Agrobacterium*-mediated transformation and regeneration of *Poplus*. *Mol. Gen. Gent*. **206**: 192-199.

Gardner, R. C. (1993). Gene transfer into tropical and subtropical crops *J. Scientia Hort*. **55**: 65-82.

Ghosh, P. K. and Chatterjee (1990). Regeneration of plants from hypocotyl derived callus tissue of jute (*C. capsularis* L.) *Cell Chrom. Res.* **13**: 26-29.

Gould, J., Devey, M., Hasegawa, O., Ulim, E., Paterson, G. and Smith, R. (1991). Transformation of *Zea mays* using *A. tumefaciens* and shoot apex. *Plant Physiol.* **95**: 426-434.

Hoque, M. A. (2001). Individual chromosome identification through karyotype analysis and production of tissue culture regenerants in garlic. M. Sc. Thesis, BAU, Mymensingh, Bangladesh. pp. 387-390.

Hidaka, T., Omura, M., Ugaki, M., Tomiyama, M., Kato, A., Ohshina, M. and Motoyashi, F. (1990). *Agrobacterium*-mediated transformation and regeneration of citrus spp. from suspension cells. *Jpn. J. Breed.* **40**: 199-208.

Hooykaas, P. J. J. and Shilperoort, R. A. (1992). *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology.* **19**: 15-38.

Hossain, A. B. M. M., Ahmed, G., Khan, R. I., Islam, M. S. (1998). Transient GUS expression in jute (*C. capsularis* L. var D-154) explants after infection by *A. tumefaciens*. *Plant Tissue Cult.* **8**(1): 11-18.

Hossain, A. B. M. M., Ahmed, G., Begum, F., Khalequzzaman, M., Khan, M. R. I. and Islam, S. (1995). Genetic transformation of jute (*C. capsularis* L. var. D-154): Gene delivery into jute cells by using *A. tumefaciens*. Abstracts. 2nd Intl. Plant Tissue Culture Conference. December 10-12. Dhaka. p. 36.

Islam, A. S. (1981). Production of desirable jute plants through tissue culture. Rao. A. N. (Ed.) Symposium on Tissue culture of economically important plants, Singapore. pp. 159-161.

Islam, A. S. (1964). A rare hybrid combination through application of hormone embryo culture. *J. Plant Physiol.* **63**(30): 310-312.

Islam, A. S., Haque, M. M. and Seraj, L. (1992). Tissue culture and micropropagation of jute (*Corchorus* spp.). Biotechnology in Agriculture and Forestry.Vol-19. High Tech. And Micropropagation III, Bajaj YPS (Ed), Springer Verlag, Berlin, Heidelberg. pp. 505-526.

Jeon, G. A., Eum, J. S. and Sim, W.S. (1998). The role of inverted repeat (IR) sequence of the *virE* gene _expression in *A. tumefaciens* pTiA6. *Molecules and Cells* **8**: 49-53.

Kouider, M., Korban, S. S., Skirvin, R. M. and Chu, M. C. (1984). Influence of embryonic dominance and polarity on adventitious shoot formation from apple cotyledons *in vitro*. *J. Amer. Soc.*, **109**: 381-385.

Khatun, A. (2001). Varietal improvement of jute through *Agrobacterium*-mediated transformation. Bangladesh Jute Research Institute, Manic Mia Avenue, Dhaka-1207. pp.233-235.

Khatun, A., Davey, M. R., Power, J. B. and Lowe, K. C. (1994). Transformation of jute cotyledons by using Agrobacterium vectors exhilarated by Pluronic F-68. Abstracts Proceed of 18th Bangladesh Science Conference, Bangladesh Agricultural University, Mymensingh, Bangladesh, Abstract No. 66.

Khatun, A. Laour, L., Davey, M. R., Power, J. B., Mulligan, B. J. and Lowe, K. C., (1992). Stimulation of differentiation in jute cotyledons cultured with Pluronic F-68. *Plant Tiss. Cult.* **2**: 75-80.

Khatun, A. Laour, L., Davey, M. R., Power, J. B., Mulligan, B. J. and Lowe, K.C., (1993). Effects of Pluronic F-68. on shoot regeneration from cultured cotyledons and on growth of transferred roots. *Plant Tiss. Org. Cult (in press)*. pp. 53-59.

Khatun, A., Laour, L., Davey, M. R., Power, J. I. and Lowe, K. C. (1993). Stimulation of shoot regeneration from jute cotyledons cultured with non-ionic surfactants and relationship to physico-chemical properties. *Plant cell Reports. 1993.* **13**: (1) 49-53.

Khatun, A. (1993). Genetic manipulation in Jute (*Corchorus spp.*). Ph D Thesis. The Univ. of Nottingham, UK. pp.289-291.

Khatun, A. and Naher, Z. (2001-2002). Optimization of culture conditions for plant regeneration from explants of kenaf and mesta. Annual Report. Bangladesh Jute Res. Inst., Manik Mia Avenue, Dhaka, Bangladesh. pp: 57-58.

Khatun, A. (2003). Plant regeneration of *C. olitorious*. *J. Plant Bio*. **30** (2): 75-80.

Khatun, A., Naher, Z., Mahboob, S., Saha, C. K. and Bilkis, S. (2003). An efficient protocol for plant regeneration from the cotyledons of kenaf (*Hibiscus cannabinus*). *Biotechnol.* **2**: 86-93.

King, A. T., Davey, M. R., Mellor, I. R., Mulligan, B. J and Lowe, K. C. (1991). surfactant. Effects on yeast cells. Enz. *Microb Technol.* **13**: 48-153.

King, A. T., Lowe, K. C. and Mulligan, B. J. (1990). Microbial cell responces to a non-ionic surfactant.II. Effects as assessed by fluorescein diacetate uptake. *Biotech. Lett.* **10**: 873-878.

King, A. T., Davey, M. R., Mellor, I. R., Mulligan, B. J and Lowe, K. C. (1990). Effects of Pluronic F-68 on plant cells in suspension culture. *Bitech. Lett* **12**: 29-32.

Klee, H., Horsch, R., Rogers, S. (1987). *Agrobacterium*-mediated plant transformation and its further application to plant biology. *Ann, Rev. of Plant Phy.* **38**: 476-486.

Kouider *et. al* (1984). Physical and functional map of supervirulent *A. tumifaciens* tumour inducing plasmid pTiBo542. *L. Bacteriil.* **166**: 88-94.

Kumar, V., Laour, L., Davey, M. R., Mulligan, B. J. and Lowe, K. C. (1992). Pluronic F-68 stimulates growth of *Solanum dulcamara* in culture. *J. Exp. Bot.* **43**: 487-493.

Kumar, V., Laouar,L., Davey, M. R., Mulligan, B. J. and Lowe, K. C. (1991). Effects of Pluronic F-68 on callus growth and protoplast plating efficiency of *Solanum dulcamara*. *Plant Cell Rep.* **10**: 52-4.

Kumar, V., Laouar, L., Davey, M. R., Mulligan, B. J. and Lowe, K. C. (1991). Effects of Pluronic F-68 on growth of transformed roots of *Solanum dulcamara*. *Biotechnol. Lett.* **12**: 937-940.

Li, X. L., Wen, L.Y., Guo, X. M., Li, E. L. and Shi, C. M. (1989). Study on regeneration of cotton plants through somatic culture. *China Cottons 1989, No.* **6**: 13-15.

Matthysse A. G. (1983). Characterization of nonattaching mutants of *A . tumefaciens*. *Journal of Bacteriology.* **169**: 313-323.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-493.

Myers, J. M. and Simon, P. W. (1999). Regeneration of garlic callus as affected by clonal variation, plant growth regulators and culture condition over time. *Plant cell Report.* **19**(1): 32.

Nath, U. K. (2001). Study of genetic diversity and *In vitro* regeneration potentiality of groundnut. M. Sc. thesis, BAU, Mymensingh, Bangladesh. pp. 331-332.

Naher, Z., Khatun, A. Mahboob, S., Alim, M. A. and Siddique, A. B. (2003). Influence of genotypes on plant regeneration from cotyledons of *C. capsularis. Biotechnol.* **2**: 86-93.

Pal, A. and Chand, P. K. (1995). *Agrobacterium*- mediated genetic transformation of some edible amaranths. Abstracts. 2nd Intl. Plant Tissue Culture Conference. December 10-12. Dhaka. p. 40.

Paszkowski, J., Saul, M. W. and Ptrykus, I. (1989). Plant gene vectors and genetic transformation: DNA Mediated Direct Gene Transfer to Plants (Eds. Schell, J. and Vasil, I.). Cell Culture and Somatic Cell Genetics of Plants Vol. 6. Molecular Biology of Plant Nuclear Genes. Academic Press. San Diego. California. pp. 52-68.

Rao, P. S. (1985). Plant protoplast: a new tool in plant biotechnology *Bangladesh J. Bot* **18**(1): 335-336.

Rahman, S. M. Z., I-Iadiuzzaman, S., I Ishaque, M. M. and Islam, A. S. (1985). Shoot Formation in *C. capsularis* var. D-154 unorganized callus. *Bangladesh J. Bot.* **14**(2): 141-145.

Raju, M. V. S. and Mann, H. E. (1970). Regenerative studies on the detached leaves of *Echevaria elegans. Can. J. Bot.* **48**: 1887-1891.

Rao, P. S. and Chadha, M. S. (1986). Protoplast culture of some economiccally important plant. Studies on plant regeneration. Proceedings of a symposium organized jointly by the International Atomic Energy Agency and the Food and Agriculture Organization of the United Nations, 19-23 Agust, Viena. pp. 493-496.

Rugini, E., Pellegrineschi, A. Mencuccini, M. and Marriotti, D. 1991. Increase of rooting ability in the woody species Kiwi (*Actinidia deliciosa* A. Chev.) by transformation with *A.* rhizogenes rol genes. *Plant Cell Rep.* **10**: 291-295.

Saha, T., Ghosh, M. and Sen, S. K. (1999). Plant regeneration from cotyledonary explants of jute, *C. capsularis* L. *Plant cell Reports.* **18**: 544-548.

Sharma, K. K., Moloney, M. M., and Walker, J. M. (1991). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.* **8**: 238-242.

Seraj, Z. I., Sarkar, A. B. and Islam, A. S. (1992). Plant regeneration in a jute species (*C. capsularis*) and its possible relationship with glyoxalase-I. *Plant Cell Reports* **12**: 29-33.

Sheerman, S. and Bevan, M. W. (1988). A rapid transformation method for *S. tuberosum* using binary *A. tumefaciens* vectors. *Plant Cell Rep.* **7**: 13-16.

Stachel, S., Nester, E. and Zambryski, P. (1986). A plant cell factor includes *A. tumefaciens* vir gene expression. Proc. Nat. *Acad. Sci. USA.* **83**: 379-383.

Thomas *et. al* (1984). The biology of genetic transformation of higher plants by *A. rhizogenes*. **In**: Puhler, A. (Ed.) Molecular Genetics of The Bacteria - Plant-Interactions, Springer-Verlag, Berlin. pp. 248-258.

Tewari, A. S., Minakshi, S. Rangaswamy, N. S. and Sethi, M. (1999). Somatic embryogenesis and plant regeneration in three cultivars of white jute. *Phytomorphology.* **49**(3): 303-308.

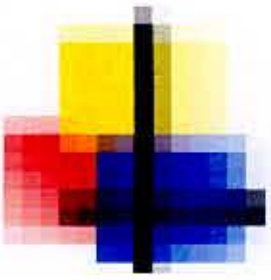
Torisky, R. S., Kovacs, L., Avdiushko, S., Newman, J. D., Hunt, A. G. and Collins, G. B. (1997). Development of a binary vector system for plant transformation based on supervirulent *A. tumefaciens* strain Chry5. *Plant Cell Reports.* **17**: 102-108.

Uematsu, C., Murase, M. Ichikawa, H. and Imamura, J. (1991). *Agrobacterium*-mediated transformation and regeneration of Kiwi fruit. *Plant Cell Rep.* **10**: 286-290.

Umbeck, P. Johnson, G. Barton, K. and Swain, W. (1987). Genetically transformed cotton (*G. hirsutum* L.) plants. *Biotechnology.* **5**: 263-266.

Wang, K., Herrera-Estrella, L., Van Montagu, M. and Zambbryski, P. (1984). Right 25-bp terminus sequence of the nonpaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell.* **38**: 455-462.

Zupan, J. R. and Zambryski, P.C. (1995). Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiology.* **107**: 1041-1047.



APPENDICES

APPENDICES

Appendix I. Constituents of stock solution for MS (1962) medium

a) Macronutrients	Concentrations (mg/l)
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
b) Micronutrients	Concentrations (mg/l)
$MnSO_4.4H_2O$	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
Na_2-EDTA.$2H_2O$	37.30
c) Iron source	Concentrations (mg/l)
$FeSo_4.7H_2O$ (Added directly as powder)	27.80
d) Organic solvent	Concentrations (mg/l)
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine acid	0.50
Thymine	0.10
Myo-inositol	100

Appendix II. Analysis of variance of the data of *C. olitorius* on no. of germinated seeds and percent of seed germination

Sources of variation	Degree of freedom	Mean squares	
		No. of germinated seeds	Percent of seed germination
Variety (A)	3	5.667**	251.904**
Error	9	0.611	27.140

** : Significant at 0.01 level

Appendix III. Analysis of variance of the data of *C. olitorius* on no. of germinated seeds and percent of seed germination on agar and cotton-supported medium

Sources of variation	Degree of freedom	Mean squares	
		No. of germinated seeds	Percent of seed germination
Treatment (A)	3	15.729**	699.054**
Error	9	0.340	15.123

** : Significant at 0.01 level

Appendix IV. Analysis of variance of the data of *C. olitorius* on no. of regenerated cotyledons, percent of regenerated cotyledons and average no. of shoots per cotyledon on different conc. of BAP

Sources of variation	Degree of freedom	Mean squares		
		Number of regenerated cotyledons	percent of shoot producing Cotyledons	Average no. of produced shoots by each cotyledon
Treatment (A)	9	6.815**	1893.119**	46.660**
Error	18	0.181	50.408	0.726

** : Significant at 0.01 level

Appendix V. Analysis of variance of the data of *C. olitorius* on no. of regenerated cotyledons, percent of regenerated cotyledons and average no. of shoots/cotyledon on different conc. of $FeSO_4$

Sources of variation	Degree of freedom	Mean squares		
		No. of regenerated cotyledons	percent of shoot producing Cotyledons	Average no. of produced shoots by each cotyledon
Treatment (A)	9	7.541**	2094.693**	55.364**
Error	18	0.152	42.186	0.274

** : Significant at 0.01 level

Appendix VI. Analysis of variance of the data of *C. olitorius* on no. of regenerated cotyledons, percent of regenerated cotyledons and average no. of shoots/cotyledon on different conc. of Pluronic F-68

Sources of variation	Degree of freedom	Mean squares		
		No. of explant showing shoot	Percent of shoot producing cotyledons	Average no. of produced shoots by each cotyledon
Treatment (A)	11	0.311**	81.380**	11.970**
Error	22	0.242	58.240	0.235

** : Significant at 0.01 level

Appendix VII. Analysis of variance of the data of *C. olitorius* on no. of regenerated cotyledons, percent of regenerated cotyledons and average no. of shoots/cotyledon for *Agrobacterium*-mediated transformation

Sources of variation	Degree of freedom	Mean squares		
		No. of explant showing shoot	Percent of shoot producing cotyledons	Average no. of shoots/cotyledon
Treatment (A)	1	0.050**	13.894**	0.512**
Error	9	0.272	75.648	0.017

** : Significant at 0.01 level