INTRODUCTION OF SALT TOLERANT GENE IN WHITE JUTE THROUGH *AGROBACTERIUM* VECTOR

By

MD. NURUL AMIN

REGISTRATION NO. 01141

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, 2009

Approved by:

(Dr. Asma Khatun)	**(Dr. Md. Shahidur Rashid Bhuiyan)**
Chief Scientific Officer	**Professor**
Bangladesh Jute Research Institute	**Dept. of Genetics and Plant Breeding**
Supervisor	**Sher-e-Bangla Agricultural University**
	Co-Supervisor

(Dr. Firoz Mahmud)

Chairman

Examination Committee

Government of the People's Republic of Bangladesh

Bangladesh Jute Research Institute

Manik Mia Avenue, Dhaka-1270.

Phone : 9110868,
8121931-2

Fax : 8802-9118415

E-mail: bjri@bdmail. net

CERTIFICATE

This is to certify that thesis entitled, **"INTRODUCTION OF SALT TOLERANT GENE BY *AGROBACTERIUM* IN WHITE JUTE (Corchorus capsularis)"** submitted to the Faculty of Agriculture, **Sher-e-Bangla Agricultural University**, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN GENETICS AND PLANT BREEDING,** embodies the result of a piece of bona fide research work carried out by **MD. NURUL AMIN,** Registration No.:03- 01141 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

Dated :
Dhaka, Bangladesh

.....
(Dr. Asma Khatun)
Supervisor

Dedicated to
My
Beloved Parents

ACKNOWLEDGEMENTS

All praises gratitude are due to the Almighty God, the great, the gracious, merciful and supreme ruler the universe to complete the research work and thesis successfully for the degree of Masters of Science in Genetics and Plant Breeding.

The author expresses the deepest sense of gratitude, sincere appreciation and heartfelt indebtedness to his reverend research supervisor Dr. Asma Khatun, Chief Scientific Officer, Bangladesh Jute Research Institute, Dhaka for her scholastic guidance, innovative suggestion, constant supervision and inspiration, valuable advice and helpful criticism in carrying out the research work and preparation of this manuscript.

The author deem it a proud privilege to acknowledge his gratefulness, boundless gratitude and best regards to his respectable co-supervisor Professor Dr. Shahidur Rashid Bhuiyan, Department of Genetics & Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for his valuable advice, constructive criticism and factual comments in upgrading the research work.

It is a great pleasure and privilege to express him profound gratitude and sincere regards to Firoz Mahmud, Chairman of Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka, for his help, criticism, suggestions and provisions of facilities and supports needed to undertake this research work.

Special appreciation and warmest gratitude are extended to Dr. Md. Sarowar Hossain, Professor of Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka and profound gratitude to Professor Abu Akbar Mia, Proffessor,

Department of Genetics and Plant Breeding, SAU, Dhaka for his enormous help, guidance and suggestions during the research period.

Heartful thanks and appreciation are due to Chandan Kumar Saha, Principal Scientific Officer and Md. Golam Mostafa, Senior Scientific Officer, Bangladesh Jute Research Institute, Dhaka for their kind cooperation, important suggestions and precious comments on the study, which smother the way of conducting the research work.

The author humbly thankful to Md. Shahadat Hossain, Scientific Officer, Bangladesh Jute Research Institute, Dhaka for his cordial cooperation during the experimental period. The author feel much pleasure to convey the profound thanks to his friends, Sharif, Koli, Sujay,,Sajoy, Rana,Faruk,Milon bhai and other well wishers for their cooperation, cheerfulness and help during the on going of the research. He particularly thankful to Hasem bhai, Taher bhai, Jalil bhai and Shafiq bhai who heavily encourage him to undertake and complete this research and thesis work.

The author thankfully remembers the students of the genetics and plant Breeding for their cooperation in the entire period of study. He also feels pleasure to all stuffs and workers of Genetics and Plant Breeding Department, SAU for their valuable and sincere help in carrying out the research work.Eventually, the author is ever grateful and expresses her special appreciation and indebtedness to his beloved parents whose sacrifice, inspiration, encouragement and continuous blessing paved the way to his higher education. He is also grateful to his brothers, sister, grandmother, and other relatives who continuously prayed for his success and without whose love, affection inspiration and sacrifice this work would not have been completed.

Dated: December, 2009
Place: SAU, Dhaka.

The Author

LIST OF ABBREVIATIONS

Symbols	Acronyms
%	Percentage
°C	Degrees Celsius
0.1 N	0.1 Normal
ANOVA	Analysis of variance
IAA	Indol acetic acid
BAP	6-benzyl amino purine
BBS	Bangladesh Bureau of Statistics
CaMV	Cauliflower Mosaic Virus
cm	Centimeter
CIP	International Potato Centre
CV	Coefficient of variation
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)
FAO	Food and Agriculture Organization
Fig.	Figure
g	Gram
gl^{-1}	Gram per liter
GDP	Gross Domestic Product
GUS	β-glucuronidase
ha	Hectare
HCl	Hydrochloric acid
$HgCl_2$	Mercuric Chloride
hrs.	Hours

LIST OF ABBREVIATIONS (Contd.)

Symbols	Acronyms
IRRI	International Rice Research Institute
J.	Journal
LB	Left border
mgl^{-1}	Milligram per liter
ml	Mili liter
MS	Murashige and Skoog
MSO	Hormone free Murashige and Skoog
Na_2-EDTA	Sodium salt of ferric ethylene diamine tetraacetate
NAA	α-napthelene acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
No.	Number
NOS	Nopaline synthase
nptII	Neomycin phosphotransferase II
NS	Non significant
pH	Negative logarithm of hydrogen ion concentration (-log $[H^+]$)
req.	Required
RB	Right border
Spp.	Species (plural)
t	Ton
T-DNA	Transfer DNA
TK	Taka
UK	United Kingdom
USDA	United States Department of Agriculture
UV	Ultra violet
V	Volt

LIST OF ABBREVIATIONS (Contd.)

Symbols	Acronyms
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X-gluc	5-bromo-4-chloro-3-indoyl- β -D-glucuronide.
Y	Year(s)
YMB	Yeast Mannitol Broth
µg	Microgram

CONTENTS

CHAPTER / SL. NO.	TITLE	PAGE NO.
	ACKNOWLEDGEMENTS	V
	ABBREVIATION	VII
	CONTENT	X
	LIST OF TABLES	XV
	LIST OF FIGURES	XVI
	LIST OF PLATES	XVIII
	LIST OF APPENDECES	XIX
	ABSTRACT	XX
CHAPTER I	INTRODUCTION	1
CHAPTER II	REVIEW OF LITERATURE	6
2.1	*In vitro* Plant Regeneration of jute	7
2.1.1	Concept of tissue culture	7
2.1.2	Tissue culture of jute	9
2.1.2.1	*In vitro* seed germination	9
2. 1.2.2	Callus induction	10
2.1.2.2.1	Effect of explants	10
2.1.2.2.2	Effects of growth regulators	11
2.1.2.2.3	Shoot regeneration	12
2.1.2.2.4	Effect of pH on plant regeneration	13
2.1.2.2.5	Influence of surfactants on plant regeneration	13
2.2	*Agrobacterium*-Mediated Genetic transformation in jute	14
2.2.1	Concept of Genetic Transformation	14
2.2.2	*Agrobacterium tumefaciens* - as a Vector	15
2.2.3.	*Agrobacterium tumefaciens* T- DNA Transfer Process	18
2.2.4	The need for genetic transformation of jute	20
2.2.5	Progress in jute transformation	21
2.2.6	Potential Improvement of Jute by Biotechnological Approach	21

2.2.7	Genetic transformation in jute	22

CHAPTER III	MATERIALS AND METHODS	PAGE NO.
3.1	Experimental Materials	26
3.1.1	Plant materials	26
3.1.2	*Agrobacterium* strain and plasmids	26
3.1.3	Glyoxalase I (GLY-I) gene	26
3.2	Sources of the experimental materials	29
3.3	Location, time duration and year	29
3.4	Media used	29
3.4.1.1	For seed germination	29
3.4.1.2	For callus induction and shoot differentiation	30
3.4.1.3	For *Agrobacterium* culture and inoculation	30
3.4.1.4	For co-cultivation	30
3.4.1.5	For washing of explants after co-cultivation	30
3.4.1.6	For selection and regeneration	30
3.5	Methods	30
3.5.1	Preparation of stock solutions	31
3.5.1.2	Steps followed for the preparation of culture media	34
3.5.1.2.1	Preparation of MS medium	34
3.5.1.2.2	Preparation of *Agrobacterium* culture medium	35
3.5.1.2.3	Preparation of salt media	40
3.5.2	Sterilization	41
3.5.2.1	Sterilization of culture media	41
3.5.2.2	Sterilization of glassware and instruments	41
3.5.2.3	Sterilization of culture room	41
3.5.2.4	Precautions to ensure aseptic condition	41
3.5.3	Culture techniques	42

3.5.3.1	ExperimentA.1 Development of a protocol for *in vitro* seed germination for Corchorus capsularis varieties	41
3.5.3.2	Experiment A.2 *In vitro* Regeneration from explants of *Corchorus capsularis*	42
3.5.3.3	Experiment-A.3.Optimization of shoot regeneration from the explants of *C. capsularis* under different hormone treatments	43
3.5.3.4	Experiment-A.4.Influence of surfactants (Pluronic F-68) on plant regeneration from cotyledon of *C. capsularis*.	44
3.5.3.4	Experiment-A.5. Influence of $FeSO_4$ on plant regeneration from cotyledone of *C. capsularis*	45
3.5.4	Experiment-B.*Agrobacterium*-mediated genetic transformation of *C. capsularis*	45
3.5.4.1	Axenic culture	46
3.5.4.2	Explant preparation	47
3.5.4.3	*Agrobacterium* culture	47
3.5.4.4	Infection and incubation of explants	47
3.5.4.5	Co-cultivation	48
3.5.4..6	Transfer to the Nutrient Medium / Subculturing	48
3.5.4.7	GUS histochemical assay	48
3.5.4.8	Transferred to Salt media	49
3.5.4.9	Transferred to Soil	49
3.6	Recording of data	49
3.7	Statistical analysis of data	51

CONTENTS (Contd.)

Chapter/ Sl. No.	TITLE	PAGE NO.
CHAPTER IV	**RESULTS AND DISCUSSIONS**	52
4.1	Experiment-A.1- *In vitro* seed germination from *C. capsularis* varieties on agar supported and clinical cotton supported medium	52
4.1.1	Percent seed germination	52
4.1.1.1	Effect of varieties	52
4.1.1.2	Effect of media	53
4.1.1.3	Combined effect of varieties and media	53
4.1.2	Experiment-A.2.-*In vitro* regeneration from explants of *C. Capsularis*	57
4.1.2.1	Callus induction	57
4.1.2.2	Days required for shoot initiation	57
4.1.2.3	Shoot regeneration Root Induction	58
4.1.2.4	Root induction	65
4.1.3	Experiment A.3-Optimization of plant regeneration from the explants of *C. capsularis* with different concentration of BAP and IAA	66
4.1.3.1	Callus Induction	66
4.1.3.1.1	Effect of phytohormone (BAP)	66
4.1.3.1.2	Effect of phytohormone (IAA)	66
4.1.3.1.3	Combined effect of BAP and IAA	67
4.1.3.2	Shoot regeneration	70
4.1.3.2.1	Effect of phytohormone (BAP)	70
4.1.3.2.2	Effect of phytohormone (IAA)	70
4.1.3.2.3	Combined effect of BAP and IAA	70
4.1.4	Experiment A.4 -Effects of different levels of pH on plant regeneration	72
4.1.4.1	Percent shoot regeneration	72

4.1.4.2	Number of shoot /cotyledon	72
4.1.5	Experiment A.5-*In vitro* evaluation of salt tolerance	74
4.2	Experiment B -*Agrobacterium* mediated genetic transformation of *C. capsularis*	77
4.2.1	GUS histochemical assay	77
4.2.2	Effect of varieties	79
4.2.3	Selection of putative transformed cells and tissues	83
4.2.4	Transfer of Putative Transformed Shoots in Rooting Medium	85
Chapter V	**SUMMARY AND FURTHER SUGGESTIONS**	90
	REFERENCES	I
	APPENDICES	I

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
Table 1	Constituents of stock solution for MS (Murashige and Skoog, 1962) medium	31-32
Table 2	Constituents of YMB medium	35
Table 3	Component of GUS staining solution	39
Table 4	Main effect of different varieties on number of seeds germinated and percent seed germination	54
Table 5	Main effect of different media on number of seeds germinated and percent seed germination	54
Table 6	Combined effect of varieties and media on number of seeds germinated and percent seeds germination	55
Table 7	Effect of different variety on number of explant showing callus, percent callus induction and days to shoot initiation	59
Table 8	Effect of different varieties of *C. capsularis* on shoot regeneration	59
Table 9	Main effect of different hormone concentration of BAP and IAA on number of explant showing callus and percent callus induction and days required for callus induction	68
Table 10	Combined effect of BAP and IAA on number of explant showing callus, percent callus induction and days required for callus induction	69
Table 11	Main effect of different pH level on percent shoots regeneration and number of shoot	73
Table 12	Selection of explants under various salt concentrations	74
Table 13	Effect of varieties of *C. capsularis* on percent shoot regeneration and average number of shoots from Agrobacterium infected cotyledon	80
Table 14	Influence of explants on transformation as assessed through GUS histochemical assay	81

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
Figure 1	The large growths on these roots are induced by *Agrobacterium* sp.	16
Figure 2	pBI121 –Region between left border (LB) and right border (RB)	27
Figure 3	*Agrobacterium tumefaciens* LBA 4404	27
Figure 4	Large view of plasmid	28
Figure 5	Cloning strategy of *PsCIPK* sense gene in plant transformation vector	28
Figure 6	Reaction catalyzed by β-D glucuronidase	39
Figure 7	Combined effect of different concentration of BAP	71

LIST OF PLATES

PLATE NO.	TITLE	PAGE NO.
Plate 1	Seed germination of jute variety CVE-3 on culture media A. Clinical cotton-supported medium and B. Agar-supported medium	56
Plate 2	Seed germination on clinical cotton and agar based media (Tricap-1)	56
Plate 3	Cotyledon on MS +2 mg/l BAP + 0.5 mg/l IAA	60
Plate 4	Callus initiation of Tricap-1 on MS + 2 mg/l BAP +0.5 mg/l IAA	60
Plate 5	Callus initiation of CVE 3 on MS +2 mg/l BAP + 0.5 mg/l IAA	61
Plate 6	Callus initiation of CVl-1 on MS +2 mg/l BAP +0.5 mg/l IAA	61
Plate 7	Callus initiation of Tricap-1 on MS + 2 mg/l BAP +0.5 mg/l IAA	60
Plate 8	Shoot regeneration in var. of CVE-3 on MS + 2 mg/l BAP +0.5 mg/l IAA	60
Plate 9	Shoot regeneration in jute var. of Tri cap--1 on MS + 4 mg/l BAP + 0.5 mg/l IA	63
Plate 10	Shoot regeneration in jute var.of CVL-1onMS + 4 mg/l BAP + 0.5 mg/l IA	64
Plate 11	Root induction in jute var. of CVl--1 on MS + 4 mg/l BAP + 0.5 mg/l IA	65
Plate 12	Salt tolerance profile of PsGLY-I gene transformed regenerating plants at different salt concentration (Variety CVL-1)	76
Plate 13	Streak culture of *Agrobacterium tumefaciens* strain LBA 4404 plasmid pB1121	78
Plate 14	GUS positive tissues showing in leaf section of variety CVL-1-(cipk)	78
Plate 15	GUS positive tissues showing in leaf section of variety Tricap-1.	79

Plate 16	Putative transgenic shoots of var. Tricap-1 containing 50mg/l kanamycin on selection medium	82
Plate 17	Putative transgenic shoots of var. CVE-3 containing 50mg/l kanamycin on selection medium	82
Plate 18	Putative transgenic plants of var. CVE-3 containing 50mg/l kanamycin on selection medium after two months	84
Plate 19	Putative transgenic plants of var. CVL-1 containing 50mg/l kanamycin on selection medium after two months	85
Plate 20	Transgenic seedling transfered to sterilize soil	86
Plate 21	Hardening of regenerated plant (CVL-1)	87
Plate 22	Hardening of regenerated plant(Tricap-1)	88
Plate 23	Transgenic seedlings (CVL-1) Transferred to Sterilize soil	81
Plate 24	Transgenic seedlings (CVL-1) Transferred to Sterilize soil	89

LIST OF APPENDICES

APPENDIX NO.	TITLE	PAGE NO.
Appendix-I	Composition of MS medium (Murashige and Skoog, 1962)	I
Appendix-II	Analysis of variance (mean squares) for no. of seeds germinated and percent seed germination	II
Appendix-III	Analysis of variance (mean squares) for no. of explants producing callus, Percent callus induction and Days required for shoot initiation	IV
Appendix-IV	Analysis of variance (mean squares) for Percent callus infection, percent shoot regeneration and Average number of Shoot	VII

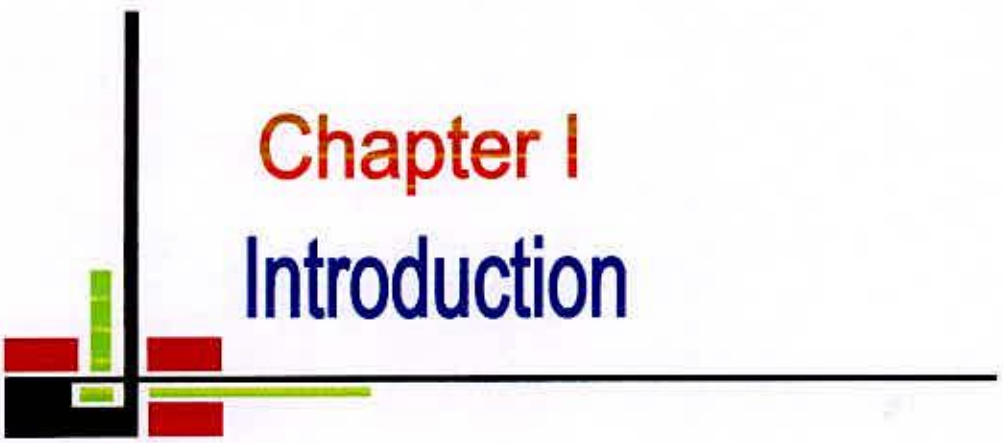
INTRODUCTION OF SALT TOLERANT GENE IN WHITE JUTE THROUGH *AGROBACTERIUM* VECTOR

By

MD. NURUL AMIN

ABSTRACT

An experiment was conducted at the Biotechnology Laboratory, Department of Genetic Resources and Seed Division, Bangladesh Jute Research Institute, Dhaka during the period of march 2008 to February 2009 on white Jute *(Corchorus capsularis)* which is one of the world's most economically important fiber crops. Genetic Engineering or recombinant DNA technology can resolve the various biotic and abiotic problems by making transgenic plant. The regeneration and transformation processes depend on optimum growth conditions, suitable explants and varieties. An attempt was made for *Agrobacterium* mediated genetic transformation in white jute varieties using gene construct conferring both salt and drought tolerance (CIPK and Gly-1)along with the marker genes. Interestingly the three varieties (CVL-1,CVE-3 and Tricap-1) showed the response of both callus induction and plant regeneration on a single formulation i.e. MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l IAA and cotyledons with attach petioles of 7-8 days old seedling .An efficient and reproducible protocol for transgenic jute plants was established by inoculating cotyledons with attached petioles and hypocotile with *agrobacterium tumefaciens* strains LBA4404,contains both salt and drought tolerant genes (CIPK and Gly-1 with selectable marker gene NptII conferring resistance to kanamycin and GUS reporter gene also. Explants were dipped to liquid culture of bacteria for one minute and then transferred to co-cultivation media for 24 hours. Shoot regeneration from *Agrobacterium* infected cotyledon was found highest in variety CVL-1 than Tricap. After co-cultivation and selection histochemical GUS assay was performed in three varieties (vars. Tricap-1, CVL-1 and CVE-3). In the transformed explants, GUS reporter gene was expressed showing blue color in the explant tissues. Among the varieties CVE-3 Showed the highest expression blue color in the explant tissues. Those transgenic plants are transferred to salt medium and soil for trial.



Chapter I

Introduction

INTRODUCTION

The worldwide awareness on environment and health is likely to provide new opportunities on jute, due to its environment-friendly characteristics. Jute is also an annually renewable energy source with a high biomass production per unit land area. It is biodegradable and its products can be easily disposed without causing environmental hazards. Jute *(Corchorus capsularis & C.olitorius)*, Kenaf *(Hibiscus cannabinus)* and Roselle *(H. sabdariffa var (Altissima)* are vegetable bast fiber plants next to cotton in importance. In the trade there are usually two names of jute, White and Tossa. *Corchorus capsularis* is called white jute and *C.olitorius* is called Tossa Jute. In India and Bangladesh roselle is usually called mesta.

Jute fibers are finer and stronger than Mesta and are, therefore, better in quality. Sacking is commonly used as packaging material for various agricultural commodities viz., rice, wheat, vegetables, corn, coffee beans etc. Sacking and hessian cloth are also used as packing materials in the cement and fertilizer manufacturing industries. Fine hessian is used as carpet backing and often made into big bags for packaging other fibres viz. cotton and wool. In recent times they are found to be a valuable aid to sound environmental management. Jute, a natural fiber that can be used in many different areas, supplementing and/or replacing synthetics, has been receiving increasing attention from the industry. Their interests focus not only on the traditional uses of jute, but also on the production of other value-added products such as, pulp and paper, geotextiles, composites and home textiles etc (BJRI. Annual Report., 2003-2004).

One of the major constrains to increase jute productivity is the non availability of land modern varieties as well as infection by fungai, bacteria, virus nematode. Farmers are encouraged to produce food grains. They push jute cultivation in marginal areas. Bangladesh is perspiring with different types of natural disasters. Because in the foreseeable future, the country is likely to be affected by the biggest ever, long lasting and global scale human induced disaster- the climate change and sea level rise (CCSLR). Bangladesh is thought to be one of the most vulnerable countries of the world to CCSLR. There are a number of environmental issues and problems those are hindering development of Bangladesh. Salinity is a current problem which is expected to exacerbate by climate change and sea level rise. Salinity intrusion due to reduction of freshwater flow from upstream, salinisation of groundwater and fluctuation of soil salinity are major concern of Bangladesh. Salinity Expansion causes due to decrease of upstream flow due to Farrakka Barrage in the upstream of the Ganges River, horizontal expansion of shrimp farms, CEP (Coastal Embankment Project), implemented during the 1960s.

The coastal region covers almost 29,000 km^2 or about 20% of the country. Again, the coastal areas of Bangladesh cover more than 30% of the cultivable lands of the country. About 53% of the coastal areas are affected by salinity. A direct consequence of sea level rise would be intrusion of salinity with tide through the rivers and estuaries. It would be more acute in the dry season, especially when freshwater flows from rivers would diminish. According to an estimate of the Master Plan Organization, about 14,000 sq km of coastal and offshore areas have saline soils and are susceptible to tidal flooding. If some 16,000 sq km of coastal land is lost due to a 45 cm rise in sea level, the salinity front would be pushed further inland. Effect of saline water intrusion in the

estuaries and into the round water would be enhanced by low river flow, sea level rise and subsidence (BBS.,2003). Agriculture is a major sector of Bangladesh's economy and the coastal area of Bangladesh is very fertile for growing crops. Increase in salinity intrusion and increase in soil salinity will have serious negative impacts on agriculture. The presently practiced jute varieties may not be able to withstand increased salinity.

In the backdrop of this situation, production of high yielding and better quality jute varieties likely salinity resistant variety is urgently felt to improve the present condition facing this natural fiber crop. Salt affected areas remain fallow owing to inadequate supply of irrigation water and also lack of salt tolerant crop varieties. Thus one might expect that the use of salinity tolerant crop varieties as well as acclimatized with saline cropping pattern would increase the net return from per unit area.

The crop has considerable commercial importance due to its diversified value-added industrial products. However, the crop demands immediate attention of plant breeders. To improve the important agronomic characters of jute, conventional breeding methods were practiced. Traditional breeding methods are employed to overcome this problem in many countries of the world including Bangladesh. However it is time consuming and has a chance of failure. Many undesired genes may also transmit.

The limitations of conventional breeding include narrow genetic base of the cultivated species, the length of time needed for successfully developing crop cultivars, the difficulty in breaking gene linkages between useful and useless traits etc. In order to produce desirable lines of jute with good growth, high fibre yield and higher biomass production an alternate technique is necessary.

Biotechnology may be an alternative way to over come this problem rapidly. For this, suitable protocol of plant regeneration and transformation is needed. Biotechnology is a recently developed novel approach, which includes a range of techniques. Together these techniques comprise a powerful technical force to produce or modify biological products according to specific objectives. Plant tissue culture, a branch of biotechnology offers an efficient method for rapid propagation, production of pathogen-free material and plant germplasm preservation.

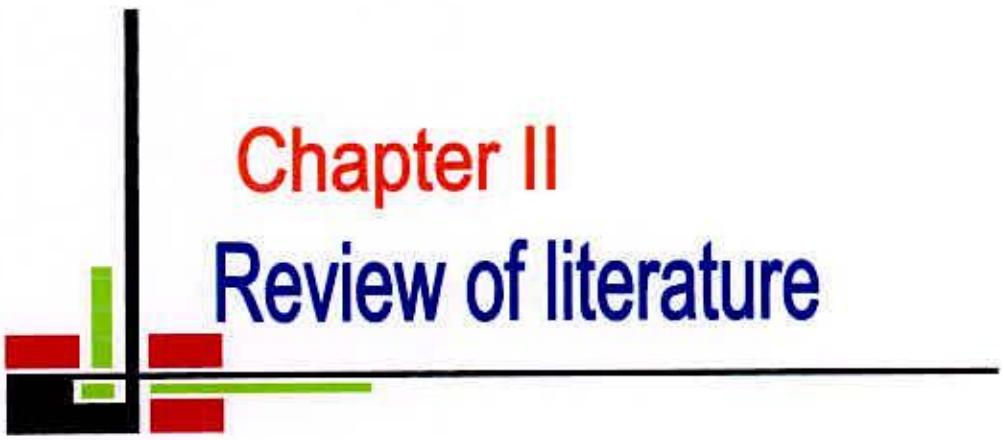
Recently, plant regeneration has already been reported from the explants of jute, which assures the exploitation of the species in tissue culture and genetic transformation system. Genetic modification of plants using recombinant DNA technologies holds the promise of increased crop productivity, product quality and reduced dependence on chemical inputs for pest control (Asano *et al.*, 1991).

Modern plant genetic engineering involves the transfer of desired genes into the plant genome and then regeneration of a whole plant from the transformed tissue. Currently, the most widely used method for transferring genes into plants is *Agrobacterium*-mediated transformation. Improvement of a species through genetic engineering includes appropriate regeneration and transformation technique. High frequency regeneration of plants from in *vitro* cultured tissues and cells is a pre-requisite for successful application of tissue culture and genetic engineering technologies for crop improvement. Both callus induction and plant regeneration from explants require the presence of appropriate combinations and concentrations of plant growth regulators in the culture media (Ehsanpour *et al.*, 2000; Fiegert *et al.*, 2000; Ahn *et al.*, 2001).

The regeneration and transformation processes depend on optimum growth conditions, suitable explants and varieties. In the present investigation attempts were made to establish a suitable regeneration and *Agrobacterium* mediated transformation protocol for jute varieties.

The specific objectives of this research program were:

- Establishment of an efficient and repeatable plant regeneration system from the explants of jute.
- Establishment of protocol for genetic transformation in jute and introduction of salinity tolerant gene through *Agrobacterium* strain.



Chapter II

Review of literature

REVIEW OF LITERATURE

Jute is the most important fibre crop of Bangladesh. The crop received much attention by a large number of researchers on various aspects of production and utilization. Improvements of crop plants like jute through conventional method require long time. Plant biotechnology now a day offers many opportunities for breeders with chances to solve certain breeding problems at cellular level. Biotechnological research on jute has been initiated in early sixties (Islam, 1964). However, output is still very limited. Recent advances in tissue culture and recombinant DNA technology have opened new avenues in transformation of higher plants, which consequently produced many transgenic plants with new genetic properties. Traditional breeding methods are employed to overcome this problem in many countries of the world including Bangladesh. However it is time consuming and has a chance of failure and many undesired genes also transmit. Biotechnology may be an alternative way to overcome this problem rapidly. Recent advances in tissue culture and recombinant DNA technology have opened new avenues in transformation of higher plants, which consequently produced many transgenic plants with new genetic properties. Establishment of an efficient plant regeneration system from the explants of jute is a prerequisite to create variability and to introduce foreign genes into this crop through genetic transformation (Khatun, 1998). The most important pre-requisite of gene transfer technique is a robust, reproducible and efficient in *vitro* regeneration protocol. However, jute is generally recalcitrant in their response to regeneration and transformation compared

to other crops. There are a number of reports on in vitro regeneration of jute (Islam *et al.* 1982, Rahman *et al.* 1985, Das *et al.* 1986, Ahmed *et al.* 1989, Saha and Sen 1992, Seraj *et al.* 1992, Khatun *et al.* 1993, Hossain *et al.* 1994, Abbas *et al.* 1997, Saha *et al.* 1999). However, the regeneration protocols described in various reports in the past need to be improved further in view of their reproducibility. Several attempts have been made to establish an efficient in *vitro* plant regeneration system from different explants of *C. capsularis* which may be applied to obtain jute transgenics of desired traits using *Agrobacterium* mediate transformation. Brief review of works done on plant regeneration and transformation of jute and allied fibres is summarized bellow.

2.1. *In vitro* Plant Regeneration of jute

2.1.1. Concept of Tissue Culture

Tissue culture is a technique of growing plant from the explant (root, shoot, cotyledon, nodule leaf, cells, tissue etc.) in an artificially prepared nutrient medium in aseptic condition where light and temperature is controlled Traditional breeding methods are employed to overcome the problems in many countries of the world including Bangladesh. However it is time consuming and has a chance of failure. Many undesired genes can also be transmitted. Biotechnology may be an alternative way to overcome this problem rapidly. For this, suitable protocol for plant regeneration and transformation is needed.

Conventional techniques are lengthy 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 (Skirvin, 1978, Larkin *et al.*, 1982). The rapid cloning of new varieties and establishment

of virus-free lines has been commercially exploited by tissue culture companies through out the world.

There are many crop species, which was successfully regenerated through plant cell culture during the last decades. For many corps like tobacco, rice and some other horticultural crops tissue culture technique has already been developed. Mo7st developed regeneration protocols contain the production of embryogenic callus from seedling explants such as cotyledon and hypocotyl sections, followed by the formation of somatic embryos with subsequent germination and conversion into mature cotton plant (Firoozabady *et al.*, 1993; Umbeck *et al.*, 1987).

Regeneration from explants like cotyledon, hypocotyls, leaves, shoot apex on defined nutrient media under sterile conditions is the basis of plant tissue culture. When explants of a plant are grown in a defined medium, an undifferentiated collection of cells arise which then developed into whole plants, this process is known as plant regeneration (Pua *et al.*, 1996; Purwati *et al.*, 1999; Debnath *et al.*, 1996).

Now-a-days, plant tissue culture techniques have been emerged as a world wide accepted concept (Ibrahim *et al.*, 1990) and opened up several new avenues for manipulation of crop plants to induce genetic changes and selection of desirable traits. Besides, plant regeneration from *in vitro* cultures is a prerequisite of many plant genetic transformation techniques.

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

2.1.2 Tissue culture of jute

In vitro plant regeneration has been quite difficult among the species *Corchorus* through tissue culture technique. It appears that jute is a notorious recalcitrant plant and regeneration from it is sporadic.

Plant regeneration has only been reported from meristematic tissue but not from totally differentiated tissue, like callus. There are reports of regeneration from cotyledon or hypocotyls derived callus, so there are usually portions of meristematic tissue left from where regeneration actually occurs.

Plant regeneration of jute from meristem (Rahman *et al.* ,1985), cotyledons (Rahman *et al.* 1985; Khatun *et al.* 1992; Ali, 1992), leaf (Islam, 1981), plumule (Das *et al.* 1996), hypocotyl (Khatun *et al.* 1992; Ghosh and Chatterjee, 1990; Seraj *et al.* 1992), apical meriatems (Rahman *et al.* 1985) and anther culture [IBFC(CAAS),1974; Islam *et al.* 1981] have been reported.

2.1.2.1 ***In vitro*** **seed germination**

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

Naher *et al.* (2003) conducted an experiment to study the seed germination percentage of varieties of *C. capsularis* (vars. CVE-3, CVL-1, D-154, CC-45, BJC-83, BJC-7370, BJC-718, BJC-2142, Tri cap-1 and Tri cap-2) on hormone free agar-solidified MS basal medium and clinical cotton-based MS liquid medium. They reported that the percentage of

seed germination among the varieties was found to be higher on cotton-based medium than the agar-based medium.

Naher *et al.* (2003) also reported that the highest percentage of seed germination was found in the variety CVE-3 (97.33%) on cotton-based medium and the lowest (86.33%) on agar-based medium.

Khatun (2001) conducted an experiment to study the germination percentage of varieties *C. capsularis* (vars. CVL-1, CVE-3, D-154 and Tricap-2) on hormone free agar-solidified MS basal media and cotton-based MS liquid medium. She reported that among the varieties, percentage of seed germination was found the highest (98.66%) on cotton-supported medium and the lowest (68.66%) on agar-supported medium in the variety CVE-3.

2. 1. 2 .2 Callus induction

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

2.1.2.2.1 Effect of explants

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

Khatun *et al.* (1992) reported cotyledon derived callus. They used phytohormones BAP and IAA with MS medium to set multiple shoots from cotyledon derived calli. Ali (1992) also recorded similar observation.

Seraj *et al.* (1992) reported that callus initiated from hypocotyls of D-154 and CVL-1 of *C. capsularis* when cultured on BAP and tyrosine fortified MS medium. They also used on antioxidant NDGA (nordihydroguaiaretic acid).

Ghosh and Chatterjee (1990) reported plant regeneration from hypocotyl derived callus tissue in MS medium of *C. capsularis*.

Islam (1981) reported that callus initiation from explants of both *C. capsularis* and *C. olitorius* and claimed that to have obtained a few shoots from leaf explants of *C. olitorius*.

2.1.2.2.2 Effects of growth regulators

Tewari *et al.* (1999) reported that 2, 4-D induced callus initiation in 100% explants when cotyledons, segments of hypocotyls and roots of white jute (*C. capsularis*) were cultured on MS medium supplemented.

Khatun (2001) cultured *in vitro* grown cotyledons (with attached petioles) of *C. capsularis* in agar solidified MS medium supplemented by 0.5 mg/l IAA and different concentration of BAP (2, 3, 4 or 5 mg/l) and noted least performance in callus induction and shoot regeneration in the combination of MS + 0.5 mg/l IAA and 2 mg/l BAP.

2.1.2.2.3 Shoot regeneration

The totipotency of somatic cells has been explained in vegetative propagation of plant species. *In vitro* studies have revealed that most

plants would differentiate shoots and roots from somatic as well as reproductive tissues.

Whole plant regeneration from cultured cells may occur either through shoot-end differentiation of plant from callus has been reported by different workers. The literatures closely related to *in vitro* regeneration of jute are cited below:

Naher *et al.* (2003) reported multiple shoot regeneration from cotyledons with attached petioles cultured on MS medium supplemented by 2 mg/l IAA and found the highest in performance CVE-3 (91%) and the lowest in Tri cap-1 (43.33%).

Khatun (2001) conducted an experiment on six varieties of jute varieties (CVl-1, CVE-3, D-154, CC-45, BJC and Tricap-2) and observed that the frequency of shoot production varied greatly among the varieties. She reported that CVE-3 (88.33%) showed the best performance in shoot regeneration.

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

Das *et al.* (1986) reported that plumule of tissue developed into multiple shoots var. D-154 of *C. capsularis* were cultured on BAP and tyrosine fortified MS media. Plant regeneration from the explants (cotyledon segments, hypocotyls segments and root segments) of *C. olitorius* (vars. O-9898, OM-1, O-72 and O-4) was difficult, but the explants of

cotyledonary petioles of all these varieties produced shoots from the cut ends (Khatun, 2001).

Two species of *Corchorus* was tested for plant regeneration (Khatun, 2001) and it was observed that plant regeneration from the explants of *C. olitorius* was very different in culture condition than *C. capsularis*. Several attempts were made by using various hormone and media combinations *in vitro* to obtain plant regeneration from various explants sources of *C. olitorius*.

Saha and Sen (1999) reported that JRC-312 showed the best shoot regenerative ability followed by JRC-212 and D-154. Tewari *et al.* (1999) conducted an experiment using three cultivars JRC-212, JRC-321and JRC-7447 for plant regeneration and only JRC-212 were regenerated.

2.1.2.2.4 Effect of pH on plant regeneration

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

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

Khatun *et al.* (1992) conducted an experiment of stimulation of differentiation in jute cotyledon cultured with Pluronic F-68. They

reported that the addition to MS-based medium of 0.1 or 0.5% (w/v) of either commercial grade Pluronic F-68 or a purified fraction obtained by passage through silica gel, stimulated shoot production from the petioles of cotyledons of *C. capsularis* vars. D154 and C134.

This effect was pronounced with C134, because of the failure of control cotyledon to differentiate into shoots in MS medium without Pluronic F-68. The implications of these results are discussed in relation to the potential value of non-ionic surfactants as additives to plant culture media for stimulating growth and differentiation.

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

2.2. *Agrobacterium* - Mediated Genetic Transformation in jute

2.2.1. Concept of genetic transformation

Genetic transformation of crop plants has been evolved, which offers the ability to introduce single new character into a plant cultivar without altering of its existing traits (Gardner, 1993). Thus, genetic transformation provides an exciting new technology to supplement traditional crop improvement programmes and together these approaches should accelerate the development of a new plant variety, which is not possible through breeding and tissue culture alone.

In all genetic 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. In this case, GUS-A (β-glucuronidase) gene and neomycin phosphotransferase II termed as npt II (kanamycin resistant), gene have been used as reporter and selectable marker genes, respectively. These reporter genes can be recognized in plant tissue with the help of selectable agents, confirming transformation of the plant tissue (through histochernical GUS assay). So, in this way, one can understand that the plant tissues subjected for transformation have really been transformed or not (Gardner, 1993).

2.2.2. *Agrobacterium tumefaciens* - as a Vector

Agrobacterium is a genus of Gram-negative bacteria that causes tumors in plants (Schell *et al.* 1997). *Agrobacterium tumefaciens* is the most commonly studied species in this genus. *Agrobacterium* is well known for its ability to transfer DNA to plants, and for this reason it has become an important tool for plant improvement by genetic engineering.

- *A.tumefaciens* causes crown-gall disease (Figure 1) in plants (Smith *et al.*, 1907).
- The disease is characterised by a tumour like growth or gall on the infected plant, often at the junction between the root and the shoot.
- Tumors are incited by the conjugative transfer of a DNA segment (T-DNA) from the bacterial tumour-inducing (Ti) plasmid (Baron *et al.*, 1997).

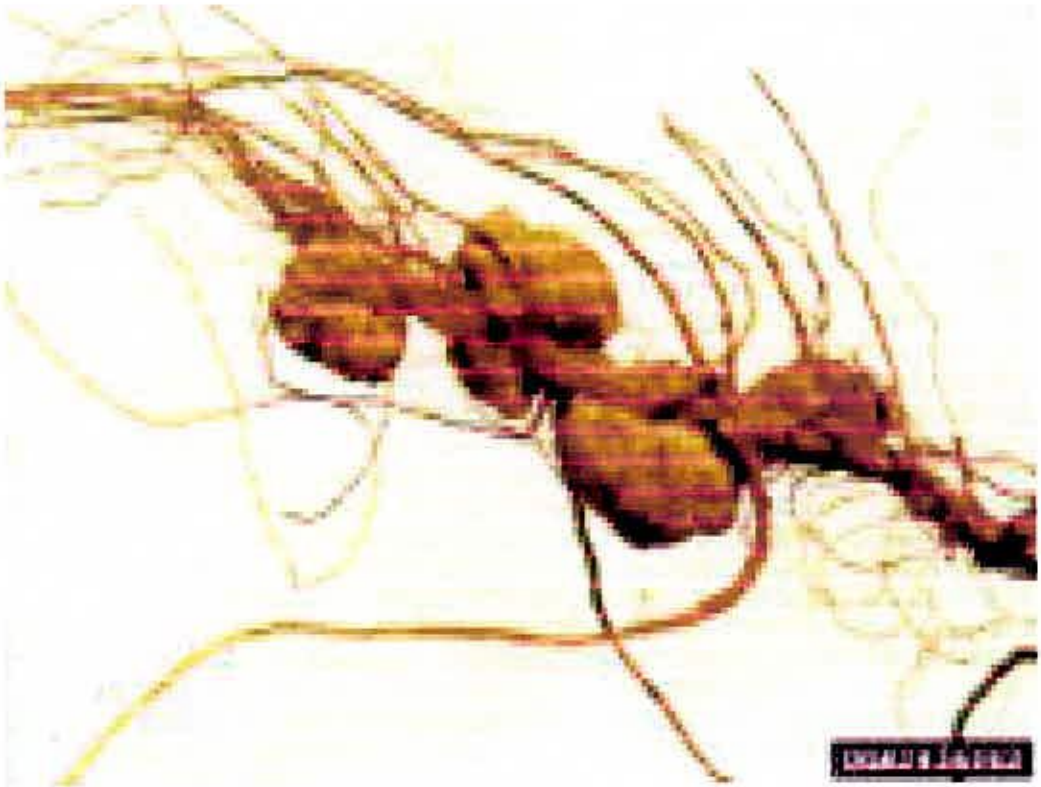


Figure 1. The large growths on these roots are galls induced by *Agrobacterium* sp.

The T-DNA carries genes for the biosynthetic enzymes for the production of unusual amino acids, typically octopine or nopaline .It also carries genes for the biosyntheis of the plant hormones, auxin and cytokinins. The ratio of auxin to cytokinin produced by the tumor genes determines the morphology of the tumor (root-like, disorganized or shoot-like).

A. tumefaciens has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-induction (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing crown gall disease (Nester *et al.*, 1984; Binns and Thomas, 1988). T-DNA contains two types of genes; the oncogenic

genes, encoding for enzymes involved in the synthesis of auxins and cytokinesis and responsible for tumor formation; and the genes encoding for the synthesis of opines.

These compounds produces by condensation between ammo acids and sugars, are synthesized and extracted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, 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).

The genes to be introduced into the plant are cloned into a plant transformation vector that containes the T-DNA region of the disarmed plasmid, together with a selectable marker (such as antibiotic resistance) to enable selection for plants that have been successfully transformed.

Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die (Das and Xie, 1998).

Agrobacterium strains contain a large mega plasmid (more than 200 kb)which plays a key role in tumor induction and for this reason it was named Ti plasmid or Ri in the case of *A. rhizogenes*. Ti 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 b; 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 organised in six operons that are essential for the T-DNA transfer (vira, vir B. VirD and virG) or for the increasing of transfer efficiency (virC and virE) (Hooykaas and Schilperoot, 1992; Zupan and Zambryski 1995, Jeon *et al.*, 1998).

Transformation with *Agrobacterium* can be achieved in two ways. Protoplasts, or leaf-discs can be incubated with the *Agrobacterium* and whole plants can be regenerated using plant tissue culture. A common transformation protocol for *Arabidopsis* is the floral-dip method: the flowers are dipped in an *Agrobacterium* culture, and the bacterium transforms the germ line cells that make the female gametes. The seeds can then be screened for antibiotic resistance (or another marker of interest), and plants that have not integrated the plasmid DNA will die (Bradly *et al.*, 1997).

2.2.3. *Agrobacterium tumefaciens* T- DNA Transfer Process

Agrobacterium tumefaciens is more than the causative agent of crown gall disease affecting dicotyledonous plants. It is also firstly the natural instance for the introduction of foreign gene in plants allowing its genetic manipulation. Similarities have been found between T-DNA and conjugal transfer systems are evolutionally related and apparently evolved from a common ancestral (Hille *et al.*, 1983).

Although the gene transfer mechanisms remain largely unknown, great progress has been obtained in practical implementation of transformation protocols for both dicotyledonous and monocotyledonous plants. Particularly important is the extension of this single-cell transformation methodology to monocotyledonous plants. This advance has biological

and practical implications. Firstly, because of advances of *A. tumefaciens*-mediated gene transfer over the direct transformation methods, which where the only way for genetic manipulation of economically important crops as cereals and legumes. Second, it has been demonstrated that T-DNA is transferred to dicot and monocot plants by an identical molecular mechanism. This confirmation implies that any plant can potentially be transformed by this method if suitable transformation protocol is established (Wood *et al.*, 2001).

The process of gene transfer from *Agrobacterium tumefaciens* to plant cells implies several essential steps:

- Bacterial colonisation
- Induction of bacterial virulence system,
- Generation of T-DNA transfer complex
- T-DNA transfer and
- Integration of T-DNA into plant genome.

T-DNA transfer process to plant cells has three important finds 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 place between the T-DNA borders can be transferred to the plant cells, no matter where it comes from. These well-established facts, allowed the construction of the first vector and bacterial strain system for plant transformation for revive (Hooykaas and Schilperoort, 1992; Deblaere *et at.*, 1985; Hamilton, 1997: Torisky *et al.*, 1997).

The *Agrobacterium*-mediated transformation protocols differ from one plant species to other and, within species, from one cultivar to other. In consequence, the optimisation of *Agrobacterium*-mediated transformation methodologies requires the considered of several factors that can be determined in the successful transformation of one species. Firstly, the optimisation of *Agrobacterium*-plant interaction on competent cells from different regenerable tissues and Secondly, the development of suiTable method for regeneration from transformed cells (Banks *et al.*, 1993).

2.2.4 The need for genetic transformation of 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-pollination. 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 the variety *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).

Since tissue culture-derived plants of a number of crops are known to produce a wide range variation (Evans and Sharp, 1986; Bajaj, 1990), it was considered worthwhile to use this technique for production of promising lines in both White and Tossa jute through somaclones. Such lines with valuable agronomic characters can be easily utilized in the breeding of desirable character (Islam *et al.*, 1992). With the development of modern biotechnology, especially the cell culture technology and gene transfer technology, it has been possible for people to introduce new traits

into plants by transforming foreign genes. It is therefore apparent that conventional means for improving jute has not been very successful. Therefore, in order to produce disease and pest resistant jute, genetic transformation with foreign genes may provide a suitable alternative.

2.2.5 Progress in jute transformation

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 *Agrobacterium 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). In another study, the stable integration of the marker gene *hpt* (conferring hygromycine resistance) was proved by surviving call in media containing hygromycin and a distinct band in PCR amplified calli DNA corresponding to inserted gene was observed. However no regeneration was nobtained from those calluses (Khan, 1998).

2.2.6 Potential Improvement of Jute by Biotechnological Approach

Resistance to several insects has been enhanced through expression of *Bacillus thuringiensis* (*Bt*) toxin genes in plants (Fishhoff *et al.,* 1987; Vaeck *et al.,* 1987; Parlaket et al., 1990, 1991) and also genes encoding protein inhibitor (Hilder *et al.*, 1989). To be effective these two types of inhibitors must be expressed in tissue specific promoters or wound inducible promoters are therefore required. The inhibitors then interfere with some aspect of digestion. Isolated *Bt* genes from promising Bt strains and inserting it into the genome of jute cultivars are the necessary

step for developing jute species resistant to hairy caterpillar. However in recent years the jute yellow mite is causing extensive damage to jute crops. Genes for proteinase inhibitors inserted into the jute genome may be effective in controlling mite, but this will require development of an efficient transformation and regeneration protocol for jute. Transgenic rice plants expressing the serine proteinase inhibitor from potato have been shown to be resistant to a major rice insect pest, pink stem borer (Duan *et al.,* 1996). Serine proteinase inhibitor expression in alfalfa has also caused to be resistant to thrips predation (Thomas *et al.*, 1984).

Various plant diseases caused by different bacterial and fungal pathogen cause remarkable yield loss. Infection of plants by these pathogen cause synthesis and accumulation of phytoalexin, production of proteinase inhibitors and an increase in activity of hydrolytic enzymes such as chitinase and glucanases.

Gene coding for such inhibitors and enzymes has been cloned from rice and other ploants. All are candidate genes for engineering disease resistance, particularly glucanases and chitinases.

Constitutive expression of chitinase in transgenic tobacco has been shown to result in an increase ability to survive in soil infected with fungal pathogen and delayed development of disease symptoms (Vierheilig *et al.*, 1993).

2.2.7 Genetic transformation in jute

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

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 *Agrobacterium 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_o transformants showed a stable inheritance of the gene to their progenies.

Haseena *et al.* (2000) reported *Agrobaterium*-mediated transformation for transfer of chitinase gene for conferring fungal resistance was attempted. Regeration of jute plantlets transformed with marker genes and chitinase gene were grown in high selection pressure for seven and two months respectively. Transformed explants were repeatedly subcultured in 1AP-containing media to reduce the chimeric nature of regenerated jute plantlets from the multiple cells in the meristematic zone. With repeated subculture, at least 30% and 40% of the regenerating plantets turned out to be albino in the 7 and 2 months old plantlets respectively.

Different attempts were made to develop *Agrobacterium*-mediated genetic trans formation in jute by Ahmed *et al.* 2000. Two strains AGL1 and LBA4404 containing binary vectors pTab7 and pZ100 were used respectively. Cotyledonary bases with petiole from 48h old germinating seedlings were used as explants. In case of pTab7 2-3 putative transgenic leafed shoot lets were regenerated from the infected

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

Gonggu (2000) reported, in China for developing an efficient transformation system for jute, factors affecting differentiation of jute explants were investigated. In a study 85% regeneration frequency was obtained. The frequency of Kan^R shoots was 4.2%. The results indicated that suitable explants were cotyledon petiole and hypocotyl and differentiation can be promoted by pre-culturing of young explants for 24 h in medium with 8 mg/1 6-BA. Shoot differentiation frequency can be improved by adding 20μM acetosyringone to co-culturing medium and survival rate of explant was increased by addition of AgN03 in selection medium.

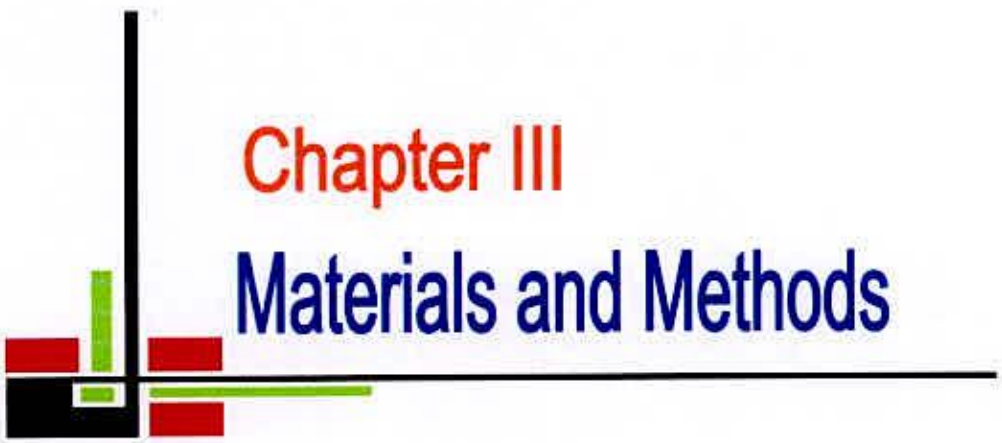
Jute variety Y-31 was transformed with the binary vector pBl-GB, which confers constitutive expression of the chitinase genes, by *Agrobacterium*-mediated method (Ruiju and Jianhua 2000).

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

Seraj *et al.* (1995) and Islam *et al.* (1996) also reported an *Agrobacterium tumefaciens* based transformation system for jute

explants. In all the above mentioned cases transformation of jute was however not confirmed by the DNA analysis.

Khatun *et al.*(1990) and later in 1993 first reported genetic transformation of jute. Hypocotyls cotyledons of *C. capsularis* and *C. olitorius* were inoculated with *Agrobacterium rhizogenes* strains 8196 and A_4 T carrying wild Ri plasmids and with strains $p^{Bin\ 121}$, R1601 and LBA9402, p^{BIN19} carrying engineered plasmids. Explants were found to be susceptible to *Agrobacterium rhizogenes* strains and produced roots from infected region. These roots were confirmed as transformed with positive opines and NPTII assays. Somatic embryos were obtained via callus formation from transformed roots in the presence of 2, 4-D, but did not regenerate into plants (khatun et al. 1990; 1993). Multiple shoot-buds were regenerated from *Agrobacterium*-infected cotyledonary petioles of *C. capsularis* (Khatun *et al.* 1994).



Chapter III

Materials and Methods

MATERIALS AND METHODS

3.1 Experimental materials

3.1.1 Plant materials

Corchorus capsularis genotypes such as Tri cap-2, CVE-3, CVL-1, D-154, Tri cap-1 and BJC-7370 were used in the present investigation to study different parameters associated with plant regeneration. Three varieties of *Corchorus capsularis* were used for jute transformation. The varieties were Tri cap-1, CVE-3 and CVL-1.

3.1.2 *Agrobacterium* strain and plasmids

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

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

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

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

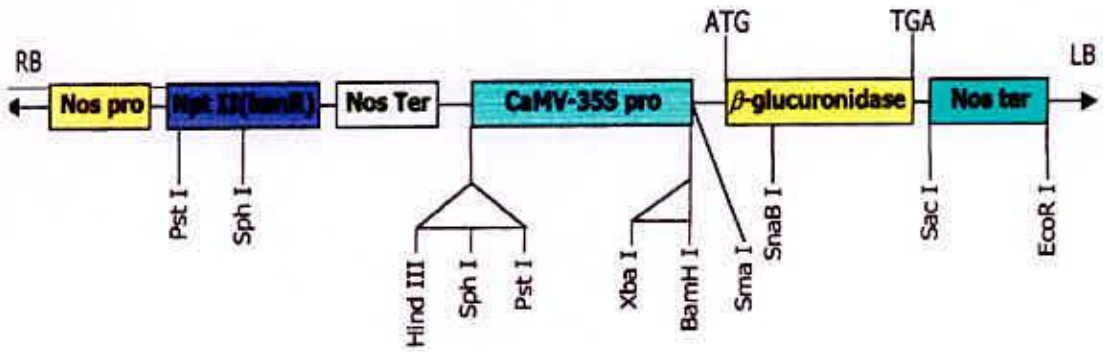


Fig 2. pBI121 –Region between left border (LB) and right border (RB)

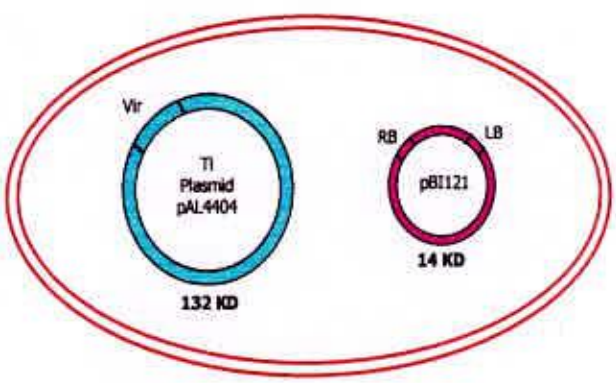


Fig 3. *Agrobacterium tumefeciens* LBA4404

3.1.3. Glyoxalase I (GLY-I) gene

The Glyoxalase I (GLY-I) gene has been chosen for introduction in sugarcane as the overexpression of this gene in the model plant tobacco has been earlier shown to impart salt, drought and heavy metal tolerance (Veena *et al*,1999).

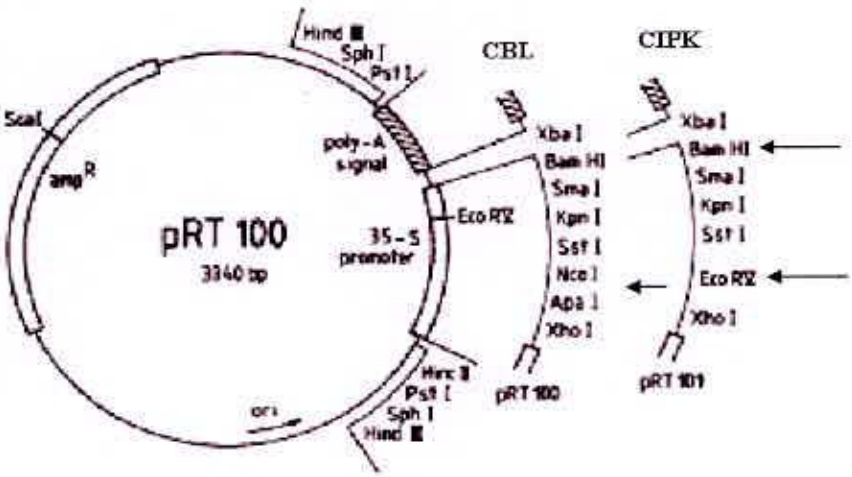


Figure 4: large view of plasmid

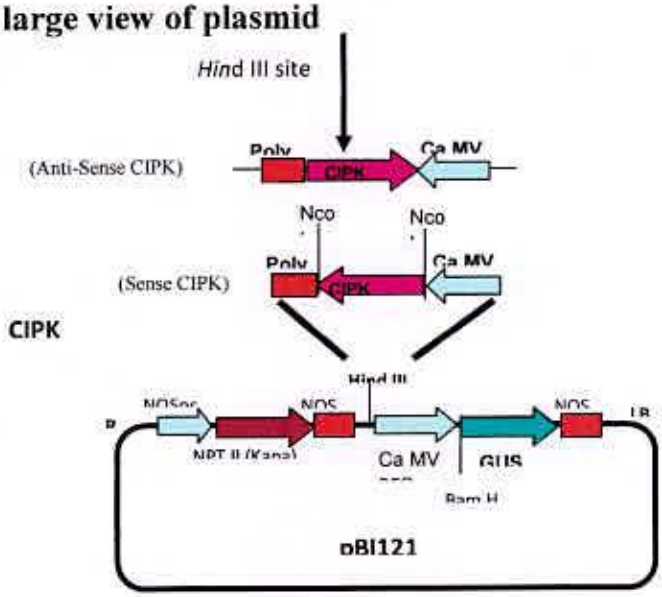


Fig 5: Cloning strategy of *PsCIPK* sense gene in plant transformation vector

Sopory and associates have been studying the role of glyoxalase system in stress tolerance. The glyoxalase system is ubiquitous in nature and consists of two enzymes, glyoxalase I and glyoxalase II, which act coordinately to convert 2-oxo-aldehydes into 2-hydroxy acids using reduced glutathione. Their primery function seems to remove methyl-glyoxal, primery substrate for glyoxalase I, a cytotoxic compound known to arrest growth and react with DNA and protein. Transgenic plants over expressing glyoxalase I showed significant tolerance to salt stress, which was correlated with degree of GLY I expression (Veena *et,al,* 1999).

3.2 Sources of the experimental materials

The materials used in the experiment were collected from Manikgonj substation of Bangladesh Jute Research Institute (BJRI), Dhaka. The strain of *Agrobacterium tumefaciens* used in this study was obtained through the courtesy of Bangladesh Sugarcane Research Institute.

3.3 Location, time duration and year

To achieve the objectives, the experiments were conducted in the Genetic Engineering Laboratory, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from February 2008 to January 2008.

3.4 Media used

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

3.4.1.1 For Seed germination

MS (Murasighe and Skoog,1962) basal medium reported by clinical cotton or agar.

3.4.1.2 For Callus induction and shoot differentiation

i. MS medium as control

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

3.4.1.3 For *Agrobacterium* culture and inoculation

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

3.4.1.4 For Co-cultivation

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

3.4.1.5 For washing of explants after co-cultivation

MS liquid medium supplemented by 500µg/ml cefotaxime

3.4.1.6 For selection and regeneration

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

3.5 Methods

For the induction of callus and plantlet regeneration in jute a number of culture media have been advocated by different scientists of which MS medium was used for conducting the present research work.

A nutrient medium (MS medium) consist of organic and inorganic salts, irons, a carbon source, some vitamins and growth regulators were used. Composition of MS medium formulated by Murashige and Skoog, (1962) is given in Appendix I.

Different steps of media preparation are described below:

3.5.1 Preparation of stock solutions

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

Table1. Constituents of stock solution for MS (Musashige and Skoog, 1962) medium

Constituents		
a) Macronutrients	Concentration(mg/l)	Concentration(g/l)10X
KNO_3	1900.00	19.00
NH_4NO_3	1650.00	16.50
KH_2PO_4	170.00	1.7
$CaCl_2.2H_2O$	440.00	4.4
$MgSO_4.7H_2O$	370.00	3.7
Na2EDTA	37.30	0.373
b) Micronutrients		100X
$MnSO_4.4H_2O$	22.30	2.23
H_3BO_3	6.20	0.62
$ZnSO_4.7H_2O$	8.60	0.86

KI	**0.83**	**0.083**
$Na_2Mo_4.2H_2O$	**0.25**	**0.025**
$CuSO_4.5H_2O$	**0.025**	**0.0025**
$CoCl_2.6H_2O$	**0.025**	**0.0025**
$FeSO_4.7H_2O$	**56.00**	**0.56 (g/l)**
c) Organic sources		(mg/l)100X
Glycine	**2.00**	**200**
Nicotinic acid	**0.50**	**50**
Pyridoxin-HCl	**0.50**	**50**
Thimine-HCl	**0.10**	**10**
Myo-inositol	**100.00**	**0.1**
d) Agar	**800.00**	**8.00**
e) Sugar	**30000.00**	**30.00**

i) Stock solution A (macro-nutrients)

The stock solution for macronutrients was made up to 10 folds (10x) of the final strength of medium in 1000 ml of distilled water. Ten times the weight of salts required per liter of the medium were weighed accurately and dissolved in 750 ml of distilled water and volume was made up to 1000 ml by further addition of distilled water. This stock solution was poured into a clean brown bottle, labeled with marker and stored in a refrigerator at 4°C for use.

ii) **Stock solution B (micro-nutrients)**

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

iii) **Stock C (Iron source)**

Required amount of $FeSO_4$ was added directly to the solution as powder.

iv) **Stock solution D (Vitamins)**

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

v) **Stock solution for hormones**

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

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

Auxin: 3-indole acetic acid (IAA)

Cytokinins: 6-benzyl amino purine (BAP)

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

3.5.1.2 Steps followed for the preparation of culture media

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

3.5.1.2.1 Preparation of MS medium

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

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

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, 10 mg of each of the hormone powder was taken in a clean beaker and dissolved in 1 ml of the appropriate solvent. The mixture was then collected in a 100 ml measuring cylinder and volume was made up to 100 ml by the further addition of distilled water. The solution was then poured into a clean volumetric flask and stored at 4°C and used for maximum period of two weeks.

3.5.1.2 Steps followed for the preparation of culture media

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

3.5.1.2.1 Preparation of MS medium

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

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

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

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

3.5.1.2.2 Preparation of *Agrobacterium* culture medium

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

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

Table 2 : Constituents of YMB medium

Mannitol	1 %
Yeast extract	0.04%
$MgS0_4.7H_20$	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 then antibiotic (kanamycin) was added at a rate of 50.0 mg/ml and separated in Petri-

dishes. When the medium became solid, the dishes were used for bacteria culture.

B) Preparation of YMB liquid medium for *Agrobacterium* suspension culture

For *Agrobacterium* suspension culture YMB liquid medium was prepared without agar. After autoclaving, the medium was cooled to 50-55^{0}C and 50.0 mg/l kanamycin was added.

C) Preparation of antibiotics (Kanamycin, and Cefotaxime)

Both of the antibiotics are soluble in water.

- Required concentration of kanamycin is 50 µg/ml
- Required concentration of cefotaxime is 500 µg/ml

D) Preparation of stock solution of Kanamycin

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

Steps:

- 250 mg kanamycin was weight by balance and taken in 5 ml. measuring flask.
- As kanamycin dissolves in water, 5 ml sterile distilled water was added in flask and dissolved by hand shaking.
- Filter sterilization was done with disposable filter sterilizer of 0.22 µm pore size and syringe.

- Distributed by 1 ml with the help of micropipette to five sterilized eppendrof tubes and stored at 4°C temperature.
- 1 ml stock contains 50 µg kanamycin.

E) Preparation of stock solution of cefotaxime

Required concentration of cefotaxime 500 µg/l for this experiment to control overgrowth of *Agrobacterium* in plant regeneration medium in washing solution. Concentration of stock solution that prepared for stock was 500µg/ml.

Total volume of cefotaxime stock solution is 2 ml. So, 2 ml solution contains 500 mg x 2 = 1000mg or 1 gm cefotaxime.

Steps:

- Cefotaxime was supplied in 1gm vial in powder form.
- As it was dissolved in distilled water,
- Two ml sterile distilled water was injected into the cefotaxime vial through sterilized syringe and dissolved by hand shaking.
- The solution was filter sterilized and stored the vial at 4°C temperature.
- 1 ml stock solutions contains 500 mg cefotaxime.

F) Preparation of 10 ml GUS staining solution

Steps:

- All necessary glassware were autoclaved
- The 8.89mg X-gluc were weighted with the help of a digital balance and care should be taken that X-gluc have high molecular weight so very minute amount is required.

- Few drops of DMSO (Dimethyl Sulphoxide) were taken in a beaker and X-gluc was added.
- Gently shaken until all of the X-gluc was dissolve.

- 200µl of Chloramphenicol was added in to the beaker.
- 10% Triton X was prepared by taken 20µl of Triton X into 200 µl of distilled. The Triton X first appeared a gel like semisolid substance, but soon dissolves if shaken gently. Then 100µl Triton X from this solution was added to the X-gluc solution.
- 2 ml of methanol was added to the solution and gently mix.
- pH of this solution was adjusted to 7.15 by adding pH buffer 10 solutions. It was noted that nearly 7 ml of buffer solution was need to adjust pH.

3.5.1.2.2.6 GUS (β-glucuronidase) Histochemical assay

GUS histochemical assay can be done after co-cultivation of infected explants as well as after culture on selection medium. Various β-D-glucuronic acid substrates are available for detection of GUS expression in *vivo* or in *vitro*. All of these substrates contain the sugar D-glucopyranosiduronic acid attached by glucosidic linkage to a hydroxyl group of a chromogenic, flurogenic, or other detectable molecule. The preferred substrate for GUS detection is 5-bromo-

4-chloro-3-indolyl-β-D-glucuronide or X-gluc (Figure 3). This colorless substrate has high extinction coefficient (making it readily detectable at low concentrations) and aqueous insolubility of the final cleavage product, dichloro-dibromoindigo (ClBr-indigo).

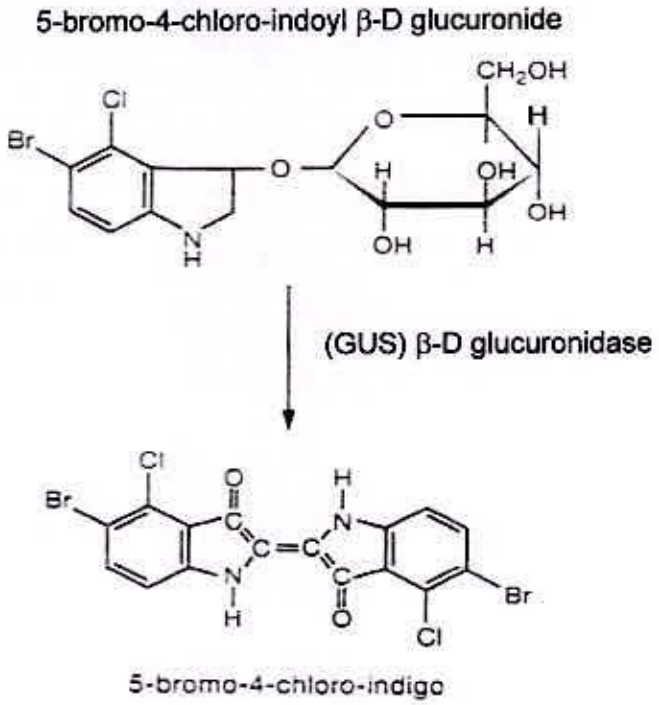


Figure 6: Reaction catalyzed by β-D glucuronidase

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

Table-3 : Component of GUS staining solution

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

pH was adjusted 7.0-8.0 by adding buffer pH-10 buffer solution up to 10 ml d.H_2O should be fixed.The co-cultivated calli, nodal segments and shoot tips were randomly selected from co-culture. The selected calli were cut into small pieces and were immersed in X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) solution and were incubated at 37^0C overnight. Similarly, the nodal segments and shoot tips were randomly selected from co-culture and were cut into pieces and sections. The pieces and sections were immersed in X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) solution were incubated at 37^0C overnight.

The expression of GUS (β-glucuronidase) gene in the plant tissue was observed . The transverse and longitudinal sections of nodal segments and part of shoot tips were observed under compound microscope and the photographs were taken with the help of a digital camera.

3.5.1.2.3 Preparation of salt media:

1 mM solution is equal to 0.0585 gm, so for preparing 25 mM/L,50 mM/L, 75 mM/L, 100 mM/L; 125 mM/L media 1.4625 gm ,2.925

gm,4.3875 mM, 5.85 gm,7.3125 gm salt is added for one litre media preparation.

3.5.2 Sterilization

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

3.5.2.1 Sterilization of culture media

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

3.5.2.2 Sterilization of glassware and instruments

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

3.5.2.3 Sterilization of culture room

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

3.5.2.4 Precautions to ensure aseptic condition

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

3.5.3 Culture techniques

3.5.3.1 Experiment-A.1. Development of a protocol for *in vitro* seed germination for *Corchorus capsularis* varieties

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

In another set of experiment, clinical cotton was used instead of agar as a supporting material for seed germination in association with MS basal medium. Clinical cotton was placed at the bottom of 100 ml flasks. Each flask contained 20 ml of hormone free MS liquid medium. Seeds of *C. capsularis* varieties were surface sterilized by immersing in absolute

alcohol for 1 minute and then in 0.1% (w/v) Mercuric Chloride for 20 minute followed by 6 washes and placed on the surface of cotton-supported MS liquid medium.

Cultures were placed in a growth room with 28^0C temperature under 1.0 Wm^{-2} of daylight fluorescent tubes with 12 hour photoperiod. Fifteen seeds were inoculated in each flask. Seven days old seedlings were used for further research work and data collection.

3.5.3.2 Exeriment-A.2. *In vitro* regeneration from explants of *C.capsularis*

The following culture methods were employed in the present investigation:

a) Axenic culture

b) Explant culture

a) Axenic culture

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

b) 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. capsularis* were taken from *in vitro* grown seedlings for this study. In this case, seedlings were allowed to develop for 7 days to make sure that the apical shoot buds were developed. Therefore, the optimum explant source, cotyledons with their attached petioles were excised from 7 days old seedlings and were cultured in 250 ml conical flasks containing 50 ml of agar-solidified MS

medium supplemented by IAA (0.5 mg/l) and BAP (2.0 mg/l). Six explants were inoculated in each culture flask. The culture flasks containing explants were placed in a growth room maintained at 28^0 C under a 1.0 Wm^{-2} of daylight fluorescent tubes with a 12 h photoperiod. The cultured flasks were checked daily to note the development of contamination. Data were recorded 6 weeks after culture.

3.5.3.3 Experiment-A.3. Optimization of shoot regeneration from the explants of *C. capsularis* under different hormone treatments

Seeds were germinated on cotton supported liquid medium following the techniques described in Section 3.5.3.1 and cotyledons with attached petiole were used as explants.

Ten explants were inoculated in each culture flask containing different treatments of BAP (0 mg/l, 1 mg/l, 2 mg/l, 3 mg/l, and 4 mg/l) and IAA (0.0 mg/l, 0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2 mg/l). The culture flasks containing explants were placed under fluorescent light in growth room with controlled temperature (28^0C). The flasks were checked daily to note the appearance of callus and shoot regeneration.

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

The following culture techniques were employed in the present study:

a) Axenic culture

b) Explant culture

a) Axenic culture

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

b) Explant culture

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

3.5.3.4 Experiment-A.5. Influence of $FeSO_4$ on plant regeneration from cotyledone of *C. capsularis.*

The following culture techniques were employed in the present study:

a) Axenic culture

b) Explant culture

a) Axenic culture

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

b) Explant culture

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

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

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

The following culture techniques were employed in the present investigation:

3.5.4.1 Axenic culture

Sterilized seeds were placed onto seed germination medium in 100 ml conical flask following the techniques described in Section 3.5.3.1. In each flask 15 seeds were inoculated. The culture was then placed in a growth room. Seven days old seedlings were used as a source of contamination-free explants.

3.5.4.2 Explant preparation

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

3.5.4.3 *Agrobacterium* culture

A single colony from previously maintained *Agrobacterium* stocks was streaked into freshly prepared Petri-dish containing YMB agar-solidified medium having 50.0 mg/l kanamycin . The Petri-dishes were sealed with parafilm and kept in room temperature for at least 48 hours. This was then kept at 4°C to check over growth of the bacteria. Such culture of *Agrobacterium* strain was thus ready to use for liquid culture. The cultures were sub-cultured regularly at each week in freshly prepared media to maintain the stock.

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

3.5.4.4 Infection and incubation of explants

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

3.5.4.5 Co-cultivation

Following infection and incubation, the explants were co-cultured on plant regeneration medium in Petri dishe containing 2mg BAP and 0.5mg IAA. Prior to transfer of all explants to regeneration medium, they were blotted dry with sterile filter papers for a short period to remove excess bacterial suspension. All the explants were maintained in co-cultivation medium for 24 hours. Petri dishes containing explants were placed under fluorescent illumination with 12 hours dark cycle at 28^0C. The intensity of light was maintained at 1000 lux.

3.5.4..6 Transfer to the Nutrient Medium / Subculturing

Following 24 hours co-cultivation, the explants were transferred to regeneration medium consisting of MS medium supplemented with 2.0 mg/l BAP, 0.5 mg/l IAAand 500 µg/ml cefotaxime. After 6-7 weeks, the regenerated shoots were transferred to hormone free MS medium with 250µg/ml cefotaxime. Amount of cefotaxime was gradually reduce in every subculturing.

3.5.4.7 GUS histochemical assay

GUS activity was detected as described by Jefferson (1987). Randomly selected co-cultivated cotyledons cultured on selective medium were used for GUS assay. Immediately after inoculation on selection medium, cotyledons were incubated in GUS staining solution at 37^0C 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 colour.

3.5.4.8. Transferred to salt media

Putatively transformed shoots were transferred to 25mM/L, 50mM/L, 75mM/L, 100mM/L; 125mM/L media.

3.5.4.9. Transferred to soil

After 4-5 times sub culturing, shoots were transferred to sterilize soil. Young, pampered seedlings that were grown either indoors or in a greenhouse will need a period to adjust and acclimate to outdoor conditions, prior to planting in the garden. This transition period is called "hardening off". Hardening off gradually exposes the tender plants to wind, sun and rain and toughens them up by thickening the cuticle on the leaves so that the leaves lose less water. This helps prevent transplant shock; seedlings that languish, become stunted or die from sudden changes in temperature. After hardening, regenerated shoots were transferred to normal soil.

3.6 Recording of data

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

a) Per cent seed germination

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

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

b) Per cent callus induction

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

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

c) Per cent 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 7-8 days after 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. Therefore, number of shoot per callus was recorded at 28 days interval and the mean was calculated using the following formula:

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

where,

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

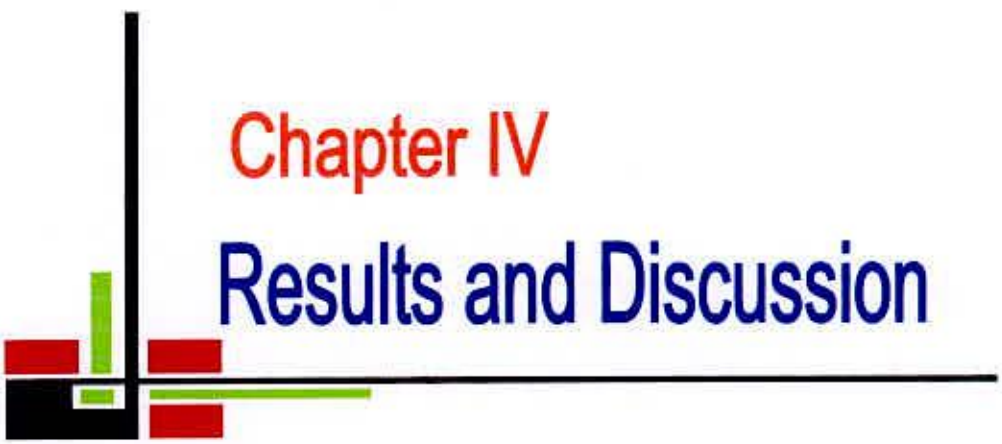
$\sum$ = summation

Xi = number of shoots/callus

n = number of observation

3.7 Statistical analysis of data

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



Chapter IV

Results and Discussion

RESULTS AND DISCUSSION

Two experiments were conducted for the present research work. In the first experiment, *in vitro* plant regeneration of two jute varieties was conducted using various hormone concentrations. In the second experiment, *Agro bacterium*-mediated genetic transformation was carried out. The results of the experiments are described along with the discussion under following heads.

4.1 Experiment-A.1. : *In vitro* seed germination from *C. capsularis* varieties on agar supported and clinical cotton supported medium

Healthy seedling production was one of the major criteria for plant regeneration from jute explants. Seeds of C. *capsularis* varieties (CVE-3, CVL-1, and Tricap-1) were germinated on both agar solidified medium and surgical cotton supported liquid medium. In the present study, number of seeds germinated and percentage of seed germination were observed.

4.1.1. Per cent seed germination

4.1.1.1. Effect of varieties

There was significant effect of varieties in per cent seed germination. The highest percentage of seed germination was found in variety CVE-3 (93.30%) and the lowest was found in Tricap-1 (79.97%) (Table. 4).

4.1.1.2. Effect of media

Per cent seed germination from the varieties *C. capsularis* was found to be higher on cotton supported liquid MS medium (85.33%) compared to agar solidified MS medium (62.16%). The result was shown in Table 5. Germination of jute seed and seedling growth in cotton supported liquid medium was found to be comparatively higher and healthier than agar solidified medium (Plate 1 and 2). This finding is supported by the findings of Khatun (2001) who reported that germination percentage was higher in cotton supported medium than the agar supported medium.

4.1.1.3. Combined effect of varieties and media

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

Table 4: Main effect of different varieties on number of seeds germinated and per cent seed germination

Variety	No. of seeds germinated	Percent seed germination
CVE -3	9.330 A	93.30 A
CVL-1	9.133 A	91.33 A
Tricap-1	7.997 B	79.97 B
CV (%)	3.93	-
LSD	0.7853	7.865

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

Table 5: Main effect of different media on number of seeds germinated and per cent seed germination

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

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

Table 6: Combined effect of varieties and media on number of seeds germinated and per cent seeds germination

Treatment		No. of seeds germinated	Percent seed germination
Varieties	**Media support**		
CVE-3	Agar	11.67 abcd	77.77 abcd
	Cotton	13..33 a	88.89 a
CVL-1	Agar	10.33 abcd	68.88 bcd
	Cotton	13.00 ab	86.66 ab
Tricap-2	Agar	10.00 bcd	66.66 cd
	Cotton	12.67 abc	84.44 abc
D-154	Agar	9.667 cd	64.44 cd
	Cotton	12.33 abcd	82.22 abcd
Tricap-1	Agar	9.667 cd	64.66 cd
	Cotton	12.00 abcd	79.99 abcd
BJC- 7370	Agar	9.33 d	62.22 d
	Cotton	11.00 abcd	73.33 abcd
CV%		13.77	13.69
LSD		2.624	17.39

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

4.1.2 Experiment-A.2. *In vitro* regeneration from explants of *C. Capsularis*

Plant regeneration through callus induction offers unique facilities of reproducible protocol as well as recovery of somaclonal variants, which can be utilized for the future crop improvement programs.

Therefore,induction of calli from cotyledonary explants and subsequent regeneration of complete plantlets is very important. The varieties viz. CVE-3, CVL-1, Tricap-1 and were used to see their performance towards plant regeneration.

4.1.2.1 Callus induction

Cotyledons (with attached petiole) of varieties of *C. capsularis* were cultured on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l IAA. Callus induction performances of all the varieties were evaluated and results are presented in Table 7.

Cotyledons (with attached petiole) started callus initiation with a change in their shape within 6 days of incubation in the all the varieties. The initial response of callus induction was exhibited by swelling of the cut ends of the cotyledons. Callus formation was completed within 8 days. Calli induced from cotyledons were mostly compact, greenish and small in size. The percentage of callus induction was highest in CVE3 (74.4%) followed by Tricap-1(65.04 %) (Plate 3 & 4).

4.1.2.2 Days required for shoot initiation

The varieties *viz.* CVE-3, Tricap-1 and CVL-1 requires shorter time to initiate shoot than other varieties. It was observed that days required for shoot initiation was minimum in CVE-3 (9.0) and maximum in CVL-1(11.00).

4.1.2.3 Shoot regeneration

The ultimate goal of *in vitro* technique is to production of free-living plantlets via shoot and root formation from the explants. The responses of different varieties towards shoot regeneration are presented in Table-8.

Shoot regeneration was the highest in CVE-3 (64.27%) followed by CVL-1 (53.30%) and Tricap-1 (50.33%) (Plate 3,4 and 5).

High responsive genotype CVE-3 for callus induction had high regeneration capacity, indicating that callus induction capacity is related to regeneration of shoot. This result correlated with the findings of Khatun (2001).

Table 7: Effect of different varieties on number of explants showing callus, percent callus induction and days to shoot initiation

Varieties	Number of explants showing callus	Percent callus induction	Days required for shoot initiation
CVE -3	11.17 A	74.40 A	9.000 A
CVL-1	9.487 A	62.82 A	11.00 A
Tricap-1	9.743 A	65.04 A	10.00 A

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

Table 8: Effect of different varieties of *C. capsularis* on shoot regeneration

Varieties	No. of explants showing shoot regeneration	Per cent shoot regeneration
CVE 3	5.800 A	64.27 A
CVL-1	4.800 B	53.30B
Tricap 1	3.800 C	50.33B

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

Plate 3. Cotyledons on MS + 2 mg/l BAP + 0.5 mg/l IAA

Plate 4. Callus initiation of Tricap-1 with attached petioles on MS + 2 mg/l BAP + 0.5 mg/l IAA

Plate 5. Callus initiation from CVE 3 from cotyledons with attached petioles on MS + 2 mg/l BAP + 0.5 mg/l IAA

Plate 6. Callus initiation from CVI-1 from cotyledons with attached petioles on MS + 2 mg/l BAP + 0.5 mg/l IAA

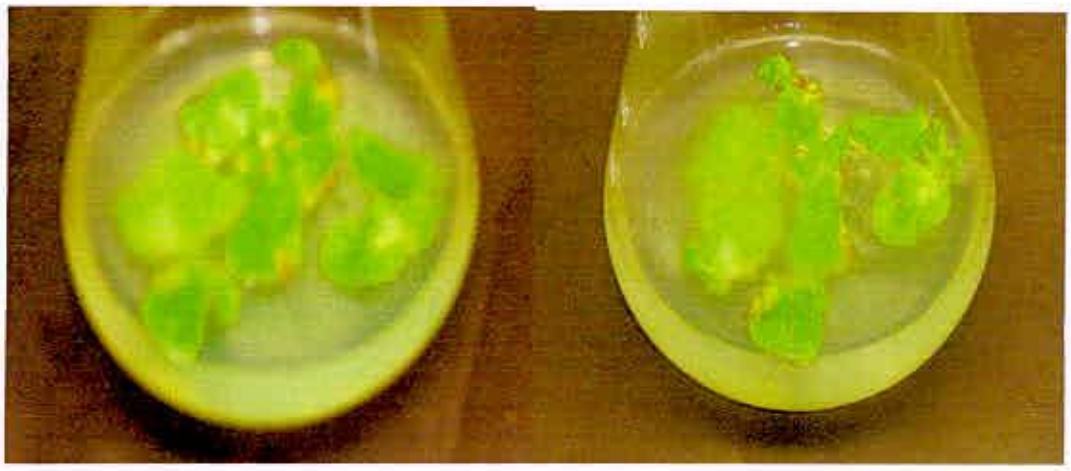


Plate 7. Callus initiation of Tri cap-1 explants on MS + 2 mg/l BAP + 0.5 mg/l IAA

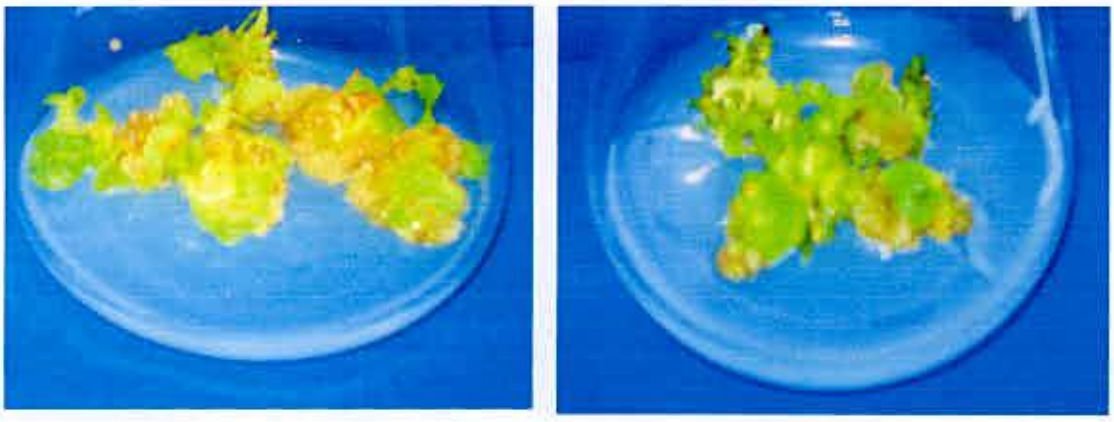


Plate 8. Shoot regeneration in var. of CVE-3 explants on MS + 2 mg/l BAP + 0.5 mg/l IAA

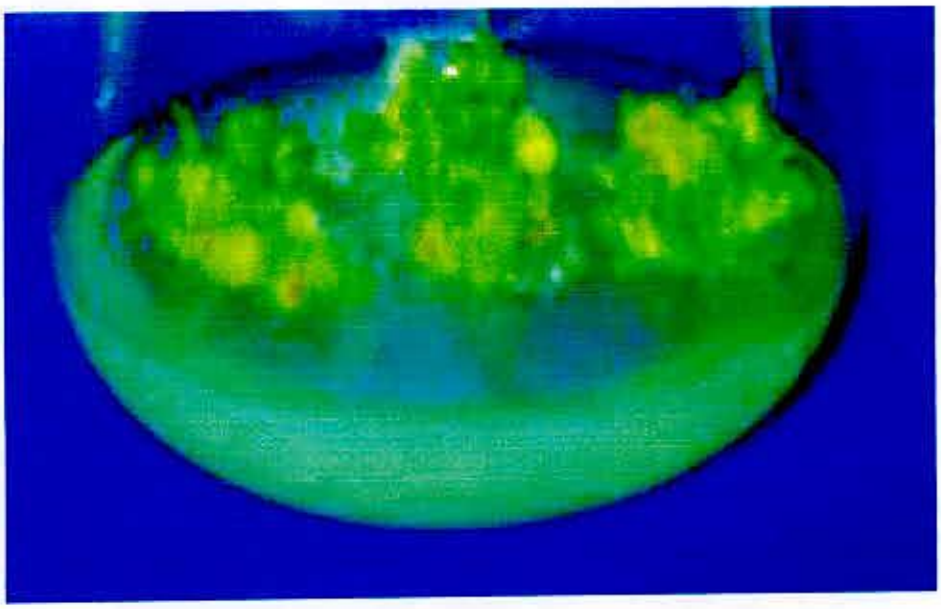


Plate 9. Shoot regeneration in Tri cap–1 on MS + 2 mg/l BAP + 0.5 mg/l IA

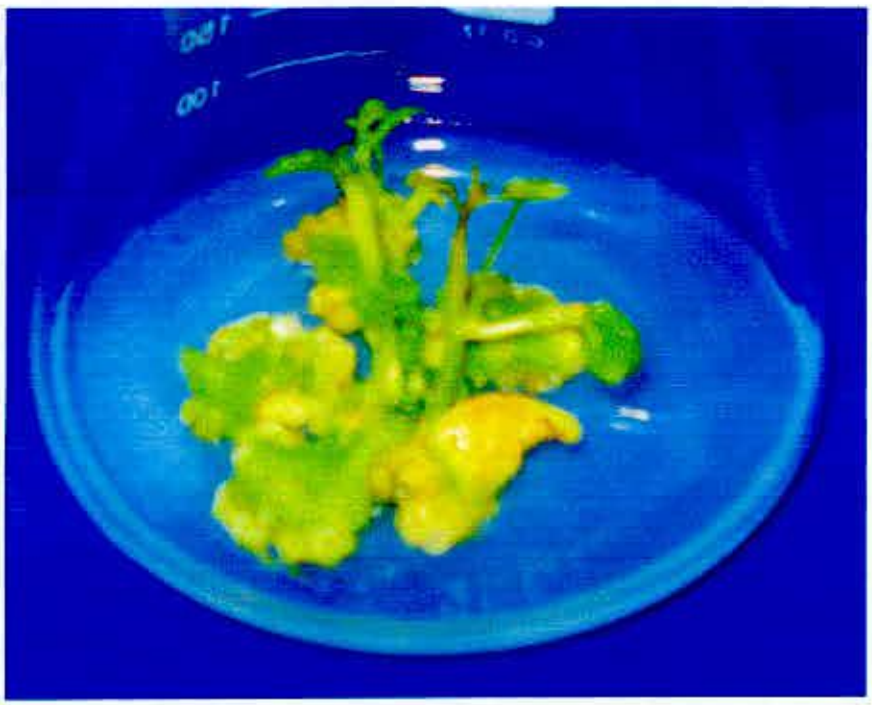


Plate 10. Shoot regeneration in jute var of CVL-1 on MS + 2 mg/l BAP + 0.5 mg/l IA

4.1.2.4. **Root induction**

The shoots regenerated from this experiment were transferred to MS medium without any hormone and all the shoots produced root (Plate 11)

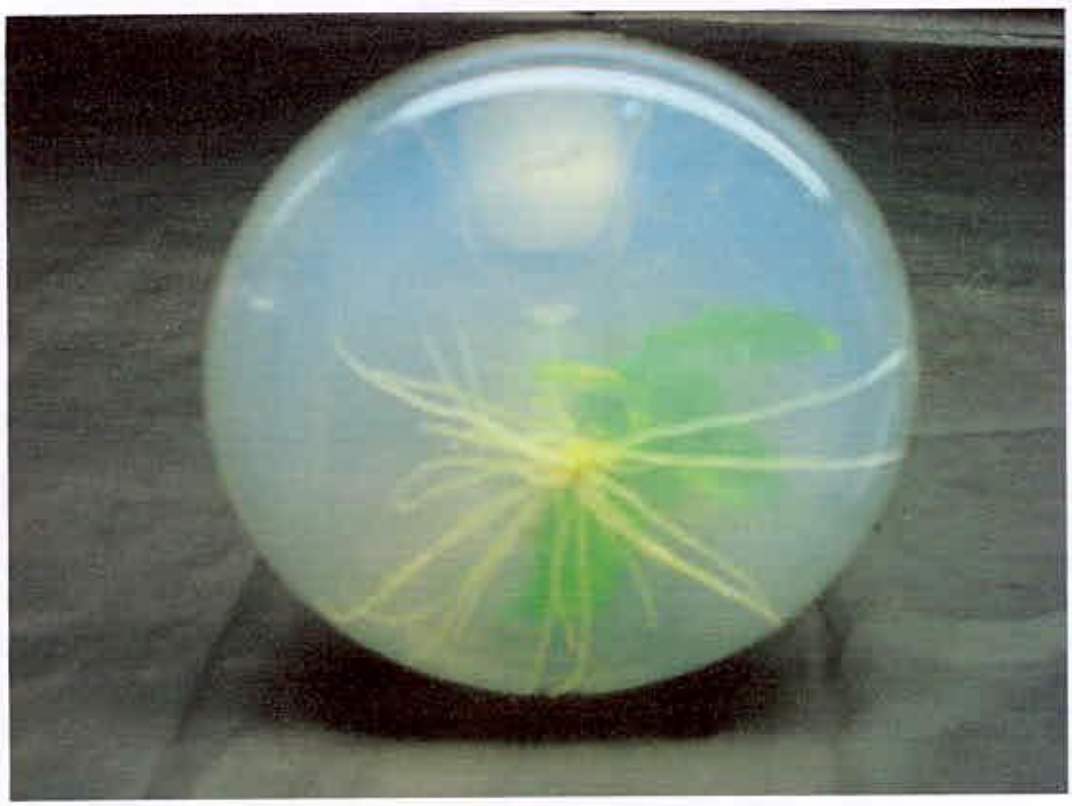


Plate 11. Root induction in var. of CVL-1 on MS medium without hormone

4.1.3 Experiment-A.3. Optimization of plant regeneration from the explants of *C. capsularis* with different concentration of BAP and IAA

In this experiment, different combination of BAP and IAA were used for callus induction and shoot regeneration using cotyledonary petioles as explants.

4.1.3.1 Callus induction

In vitro callus induction depends on a number of factors including proper concentration of growth regulators.

4.1.3.1.1 Effect of BAP

Different concentration of BAP levels showed significant variation for number of explants showing callus, percent callus induction and days required for callus induction, indicating significant differences among the concentrations of the BAP on these characters. BAP at 2 mg/l was found to be the best for all the characters (Table 7).

4.1.3.1.2 Effect of IAA

Mean value due to different concentration of IAA for number of explants showing per cent callus induction and days required for callus induction were significant, indicating the presence of variation among the concentrations used for this study. IAA at 0.5 mg/l concentration was found to be the best. Maximum number of explants showing callus (8.44) with the highest percentage of callus induction (84.40%) was found at this concentration. It was also observed that the above concentration requires shorter days to initiate callus.

4.1.3.1.3 Combined effect of BAP and IAA

Combined effect of BAP and IAA on number of explants showing callus, percent callus induction and days required for callus induction are presented in Table 9

The highest percentage of callus induction was found in the combination of MS+2 mg/l BAP+0.5 mg/l IAA (96%) followed by MS+ 2mg/l BAP+ 1.5 mg/l IAA (94%). Minimum days (6.0) required for callus induction was observed in the combination of MS+ 0.0 mg/l BAP+ 1.0 mg/l IAA.

Table 9: Main effect of different hormone concentration of BAP and IAA on number of explants showing callus and percent callus induction and days required for callus induction

Treatment	No. of explants showing callus	Per cent callus induction	Days required for callus induction
BAP (mg/L)			
0.0	5.36 c	53.60 c	7.16 a
1.0	8.32 ab	83.20 ab	8.00 a
2.0	8.96 a	89.60 a	5.72 b
3.0	8.04 ab	80.40 ab	7.44 a
4.0	7.56 b	75.60 b	7.52 a
IAA (mg/L)			
0.0	5.84 b	58.40 b	6.72 bc
0.5	8.44 a	84.40 a	6.12 c
1.0	8.12 a	81.20 a	6.88 bc
1.5	7.92 a	79.20 a	7.76 ab
2.0	7.92 a	79.20 a	8.36 a

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

Table 10: Combined effect of BAP and IAA on number of explants showing callus induction, percent callus induction and days required for callus induction

Treatments		No. of explants showing callus induction	Percent callus induction	Days required for callus induction
BAP (mg/L)	IAA (mg/L)			
0.0	0.0	-	-	-
	0.5	6.80 gh	68.00 gh	6.20 f
	1.0	7.00 fgh	70.00 fgh	6.00 f
	1.5	6.40 h	64.00 h	7.80 bcd
	2.0	6.60 gh	66.00 gh	8.60 ab
1.0	0.0	7.00 fgh	70.00 fgh	9.20 a
	0.5	9.00 abcd	90.00 abcd	6.40 ef
	1.0	8.80 abcde	88.00 abcde	7.00 cdef
	1.5	8.40 abcdef	84.00 abcdef	8.20 abc
	2.0	8.40 abcdef	84.00 abcdef	9.20 a
2.0	0.0	7.80 cdefg	78.00 cdefgh	6.60 def
	0.5	9.60 a	96.00 a	6.80 def
	1.0	9.20 abc	92.00 abc	6.40 f
	1.5	9.40 ab	94.00 ab	7.80 bcd
	2.0	8.80 abcde	88.00 abcde	8.20 abc
3.0	0.0	7.40 efgh	74.00 efgh	6.00 f
	0.5	8.40 abcdef	84.00 abcdef	6.60 def
	1.0	8.00 bcdefg	80.00 bcdefg	8.20 abc
	1.5	8.40 abcdef	84.00 abcdef	7.80 bcd
	2.0	8.00 bcdefg	80.00 bcdfgh	8.60 ab
4.0	0.0	7.00 fgh	70.00 fgh	8.80 ab
	0.5	8.40 abcdef	84.00 abcdef	7.60 bcde
	1.0	7.60 defgh	76.00 defgh	6.80 def
	1.5	7.00 fgh	70.00 fgh	7.20 cdef
	2.0	7.80 cdefgh	78.00 cdefgh	7.20 cdef

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

4.1.3.2 Shoot regeneration

4.1.3.2.1 Effect of BAP

Different concentration of BAP showed significant variations for number of explant showing shoot, regeneration per cent, shoot regeneration and days required for shoot regeneration. The responses of calli to different concentrations of BAP towards shoot regeneration are presented in Table 11.Shoot regeneration was found the highest at 2 mg/l BAP (42.23%) followed by 3 mg/l BAP (30.12%). Days required for shoot regeneration was also minimum (18.72) at 2 mg/l BAP concentration.

Number of explants showing shoot and percent shoot regeneration gradually increased with the increasing level of BAP upto 2 mg/l. Further increasing of BAP level did not show any improvement of number of explants showing shoot and per cent shoot regeneration.

4.1.3.2.2 Effect of IAA

The highest percentage of shoot regeneration was found at 0.5 mg/l IAA (35.94%) than the other concentrations. Days required for shoot regeneration was also found minimum (19.74) at this concentration.

4.1.3.2.3 Combined effect of BAP and IAA

The combined effect of BAP and IAA (Fig.7) showed that highest shoot regeneration percentage (60.66%) was recorded in MS medium supplemented with 2 mg/l BAP and 0.5 mg/l IAA followed by MS+2mg/l BAP+ 1.0 mg/l IAA (47.28%). This finding is similar to the finding of Khatun (2001) who found the best performance in shoot regeneration on the combination of MS+2 mg/l BAP+0.5 mg/l IAA. No shoot regeneration ability was found without IAA and BAP. It may be concluded that MS+2mg/l BAP+0.5 mg/l IAA combination was favourable for higher percentage of shoot regeneration.

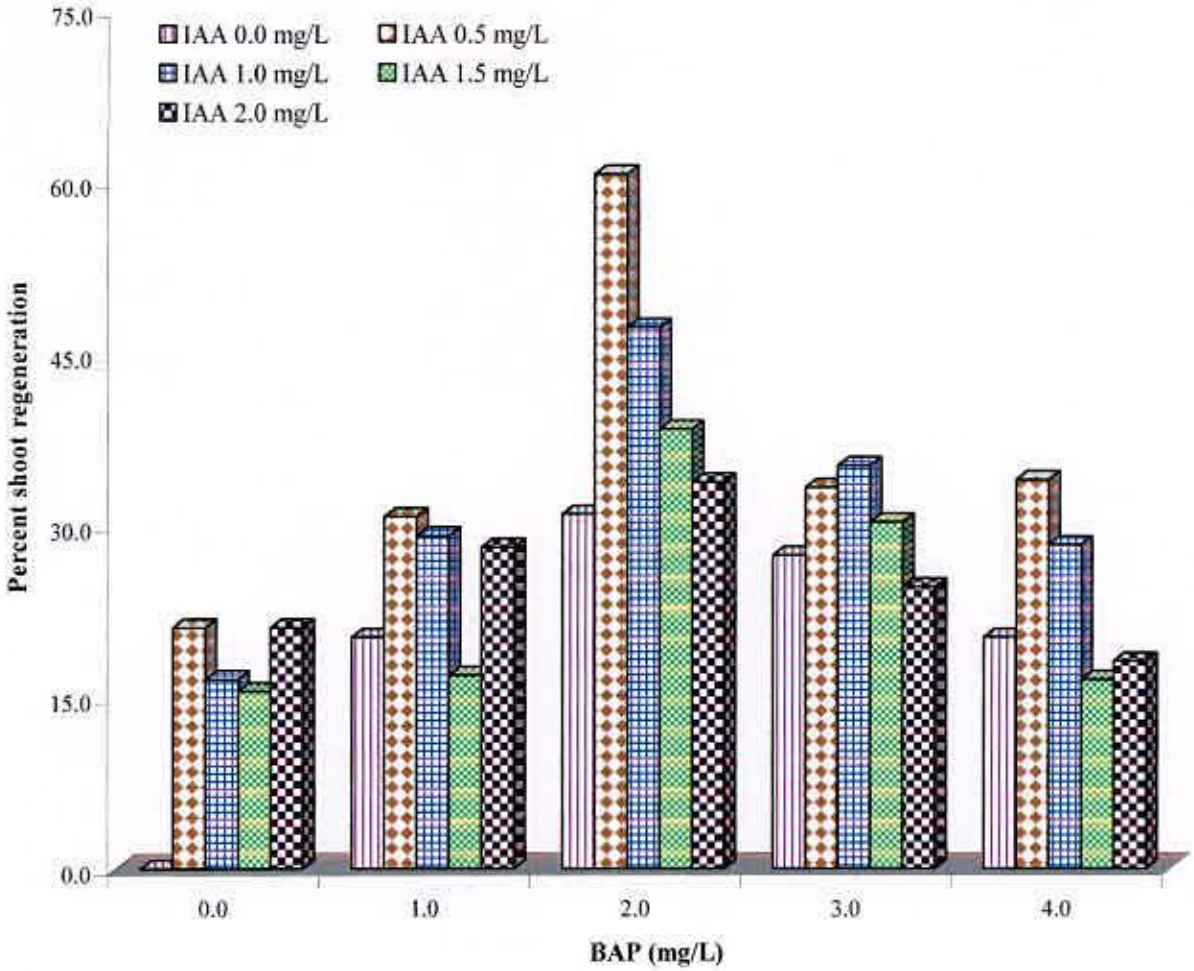


Fig. 7: Combined effect of different concentration of BAP and IAA on percent shoot regeneration

4.1.4 Experiment-A.4. Effects of different levels of pH on plant regeneration

Different varieties responded differently on various levels of pH.

4.1.4.1 Per cent shoot regeneration

It may be concluded that pH has significant effect on shoot regeneration.

4.1.4.2 Number of shoot per cotyledon

It was observed that number of shoot per cotyledons decreased as the pH level increased.

Table 11: Main effect of different pH level on percent shoots regeneration and number of shoot/cotyledon

pH levels	Per cent shoots regeneration	No. of shoot per cotyledon
4.0	35.80 c	5.60 d
4.5	41.40 bc	7.70 c
5.0	46.80 b	9.40 b
5.5	58.50 a	10.40 ab
6.0	44.80 bc	10.60 a
6.5	43.00 bc	7.40 cd
7.0	42.30 bc	6.00 d
7.5	39.50 bc	4.40 e

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

4.1.5 Experiment-A.5. *In vitro* evaluation of salt tolerance

Table 12. Selection of explants under various salt concentrations

Treatments (Salt concentration)	Number of explants assayed	Number of explants survived	Percentage of survived plants	Days of survival
25 mM	10	10	100	60
50 mM	10	9	90	30
75 mM	10	5	50	15
100 mM	10	2	20	8
125 mM	10	0	00	5

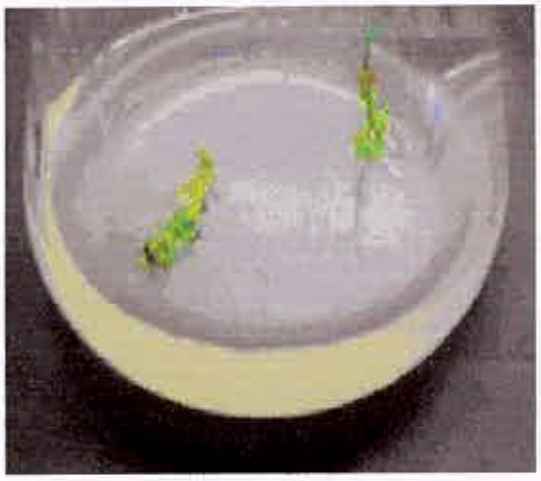
Figures followed by same letter in a column do not differ significantly

For the evaluation of salt tolerant genes transformation in both of the two jute varieties was performed using *in vitro* method under controlled conditions. MS media supplemented with NaCl salt concentrations (25, 50, 75, 100 and 125 mM) was used for salt tolerance testing of transformed plants. Transformed plants of two varieties were able to survive up to 100 mM salt concentration while the controlled plants died out at NaCl salt concentration of 50mM (Plate-12).Both of the varieties survived on (50 %) up to 75 mM salt concentrations. However, in case of 100 mM salt concentrations, the survival rate was 20 % (Table 13).The

explants were albino at 125 mM Nacl salt concentration. The survival of transformed plants at 75 mM of NaCl salt concentration indicates the transformation of desired salt tolerant gene and successful expression of the genes in the varieties CVL-1 and Tri cap. It signals the possibility of the salt tolerant variety development using recombinant DNA technology for cultivation in the saline belt of Bangladesh.

A. 25 mM NaCl salt concentration

B. 50 mM NaCl salt concentration



C. 75 mM NaCl salt concentration

D. 100 mM NaCl salt concentration

Plate 12. Salt tolerance profile of Ps GLY-I gene transformed regenerating plants at different salt concentration (variety CVL-1)

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

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

Therefore, in the present study, investigations were made to generate transgenic plant from three varieties of *C. caplusaris* (viz. Tricap-1, CVE3 and CVL1) through *Agrobacterium tumificiens* mediated transformation using cotyledons (with attached petiole) as explants.

4.2.1 Histochemical GUS (β-glucuronidase) assay

After infection of the callus explants in *Agrobacterium* suspension culture, they were transferred to co-cultivation medium. Following incubation and co-cultivation with *Agrobaaterium*, transformation ability was monitored through histochemical assay of GUS reporter gene in explants tissue. Transient GUS assay was done at the end of co-cultivation with randomly selected and inoculated explant tissue. In the GUS assay, conspicuous GUS positive (blue color) regions were detected in the explants surface (Plate.14).The detailed results of the investigation were presented in Table 14.

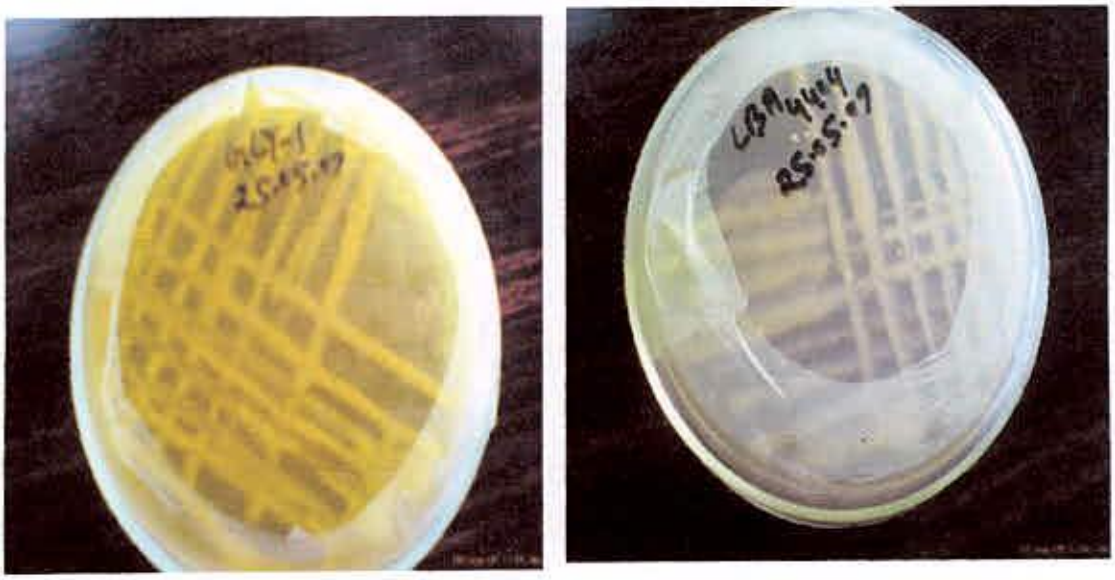


Plate 13. Streak culture of *Agrobacterium tumefaciens* strain LBA 4404 plasmid pB112l

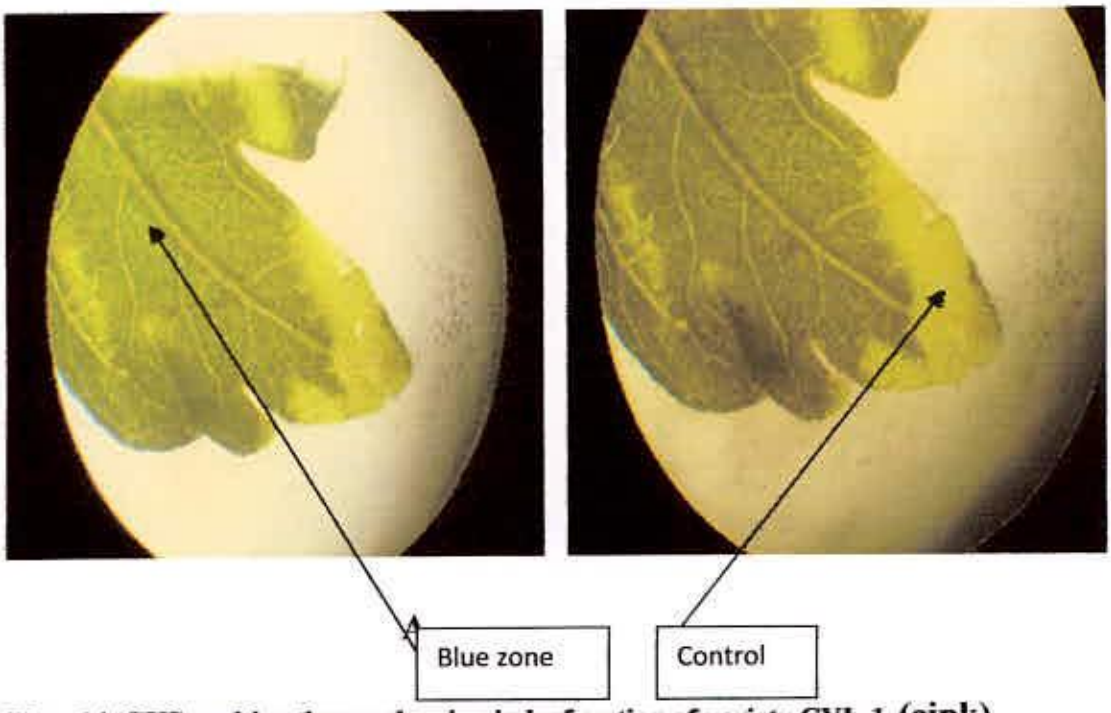


Plate 14. GUS positive tissues showing in leaf section of variety CVL-1-(cipk)

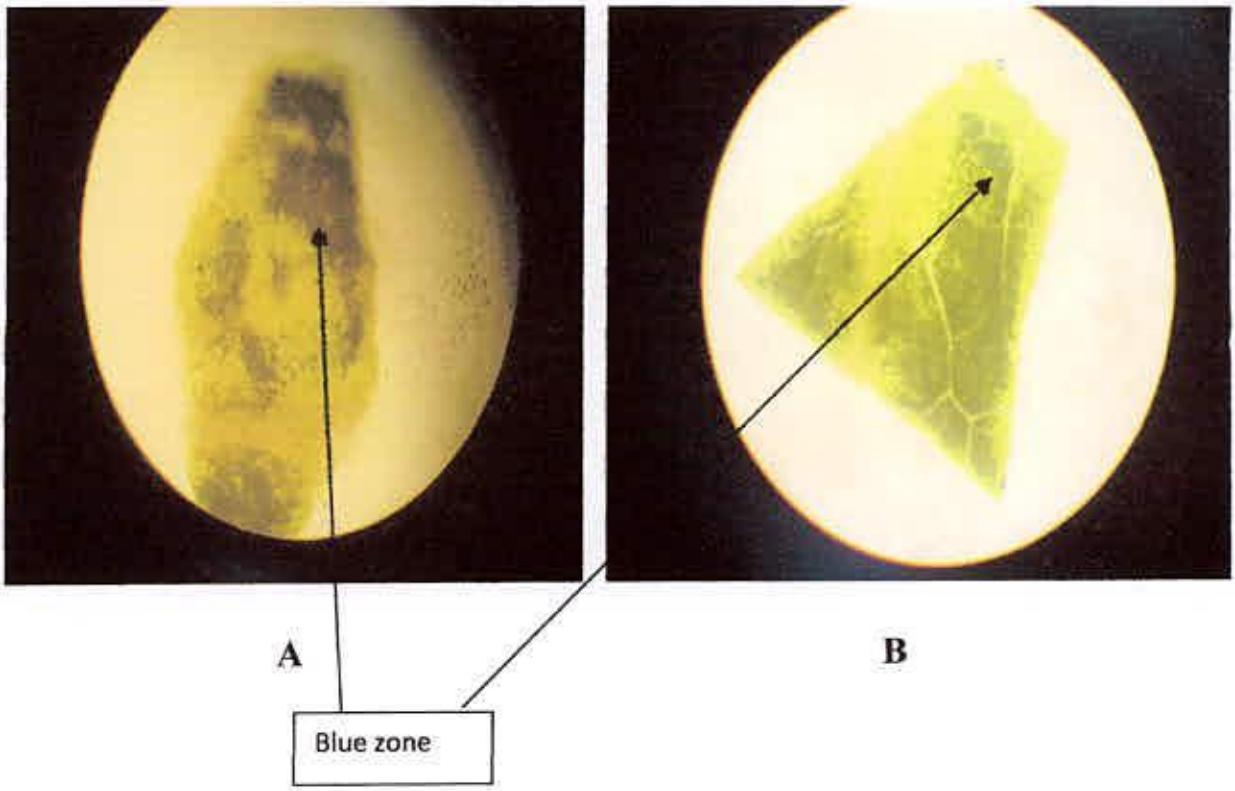


Plate 15. GUS positive tissues showing in leaf section of Tri cap-1.

4.2.2 Effects of varieties

Following *GUS* histochemical assay, it was found that all the three varieties showed positive responses towards transformation. Among the varieties, Following infection and co-cultivation with the strain LBA4404, the cotyledons attached with petioles were cultured on plant regeneration medium containing cefotaxime 500 µg/ml for shoot development. Shoot regeneration from *Agrobacterium*-infected cotyledons was found to be the highest in CVL-1 (43.00 %) followed by CVE-3 (38.67%) Table 11 (Plate 9 and 10). The average number of shoots produced by each cotyledon was also found to be the highest in CVL-1 (14.33) and the lowest in Tri cap (8.667).

Table 13. Effect of varieties of *C. capsularis* on percent shoot regeneration and average number of shoots from *Agrobacterium* infected cotyledon

Varieties	Number of cotyledons infected	Percent shoot regeneration	Average number of shoots produced by each cotyledon
CVE-3	60	38.67 AB	11.33 B
CVL-1	60	43.00 A	14.33 A
Tri cap-1	60	33.00 B	8.667 C
CV%		6.83	4.17
LSD		6.045	1.772

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

Table 14. Influence of explants on transformation as assessed through GUS histochemical assay

Variety	Number of explants infected	Number of explants assayed for GUS	Number of explants +ve for GUS	% of GUS +ve explants
CVE3	60	10	9	90.00
Tricap-1	60	10	8	80.00
CVL1	60	10	8	80.00

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

Plate 16. Putative transgenic shoots of var. Tricap-1 containing 50mg/l kanamycin on selection medium

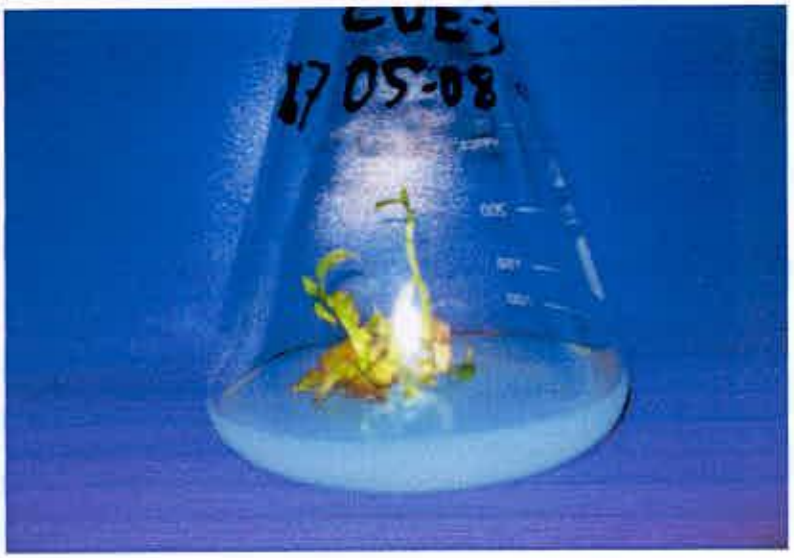


Plate 17. Putative transgenic shoots of var. CVE-3 containing 50mg/l kanamycin on selection medium

4.2.3 Selection of putative transformed cells and tissues

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

Plate 18. Putative transgenic plants of var. CVE-3 containing 50mg/l kanamycin on selection medium after two months

Plate 19. Putative transgenic plants of var. CVL-1 containing 50mg/l kanamycin on selection medium after two months

4.2.2.4 Putative Transformed Shoots Transferred to Soil

After root production, transgenic plantlets were subjected to hardening and then transferred to sterilize soil successfully and after shoot and root development it transferred to normal soil in big pot .(Plate 20, Plate 21, Plate 22, Plate 23, Plate 24).

Plate 20. Seedling transfered to sterilize soil

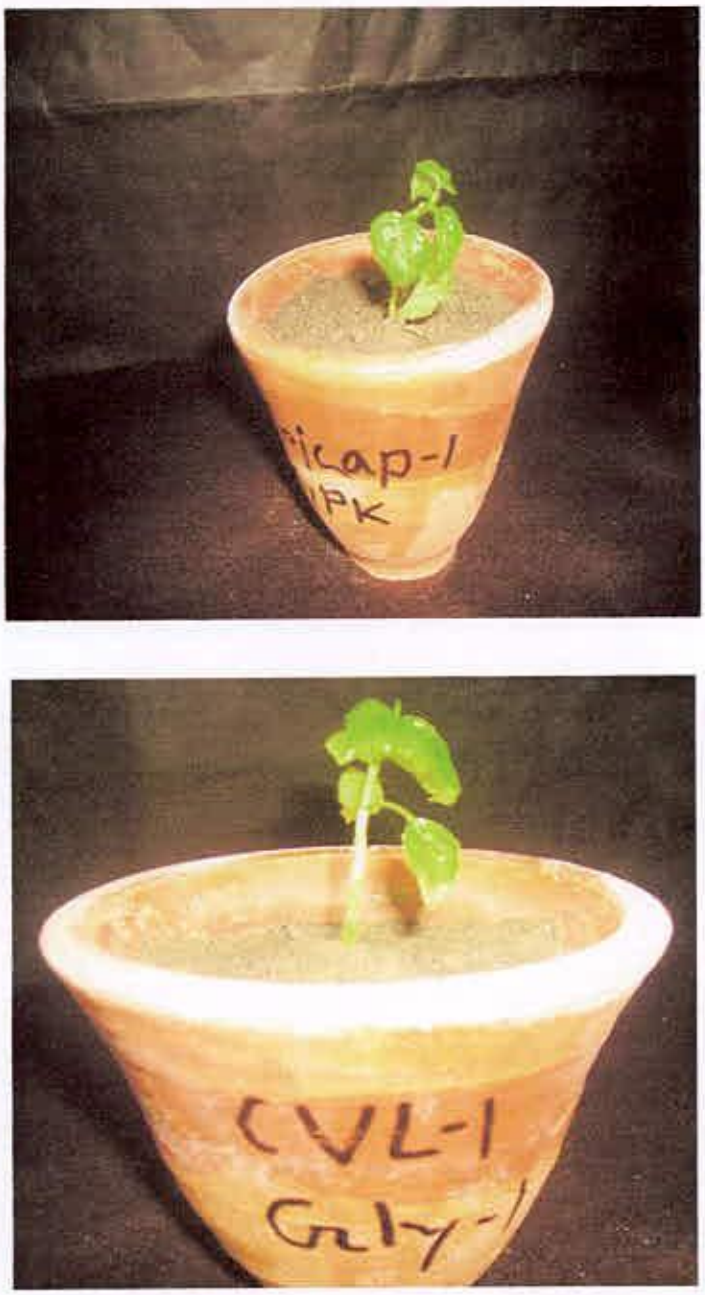


Plate 21. Transgenic seedling transfered to sterilize soil

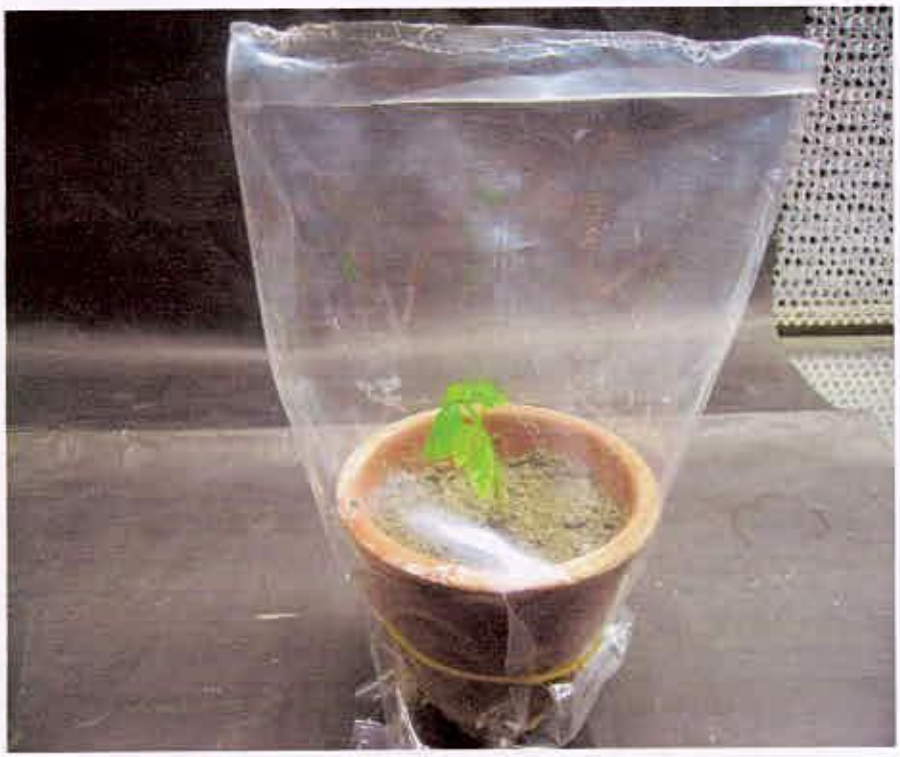
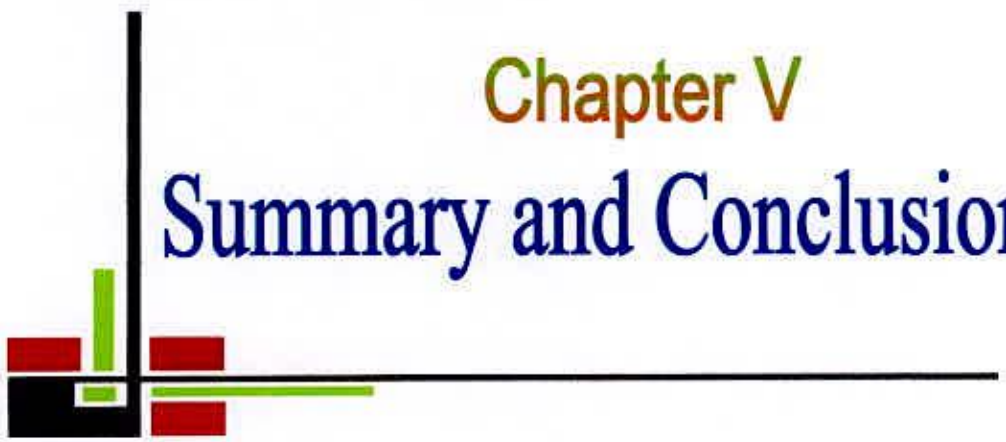


Plate 22. Hardening of regenerated plant (CVL-1)

Plate 23. Hardening of regenerated plant(Tricap-1)

Plate 24. Transgenic seedlings (CVL-1) Transferred to Sterilize soil



Chapter V

Summary and Conclusion

SUMMARY AND CONCLUSION

Two different sets of experiments were carried out in the Biotechnology Laboratory, Cytogenetic Department, Bangladesh Jute Research Institute (BJRI), Dhaka during the period 0f February 2008 to February 2009.

In the first experiment, a detailed investigation was carried out to study the seed germination, callus induction ability and subsequent plant regeneration of *Corchorus capsularis* genotypes using cotyledons (with attached petioles) as explant. In the second experiment, investigation was carried out to study the *Agrobacterium tumificiens* mediated genetic transformation of white jute.

Significant effect of varieties was found in percent seed germination. The variety Tri cap-1 was found to be the highest in germination percentage (93.3%) and the lowest was found in CVL-1 (79.967%).

In cotton-based media, the germination percentage was found to be higher (85.33%) compared to agar solidified media (62.16%). From the results of the present study it was found that cotton-based media was found to be better than agar-solidified MS medium for seed germination.

Cotyledons (with attached petioles) of *C. capsularis* varieties were cultured on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l IAA to observe their callus induction and shoot regeneration capacity. A wide range of variation in callus induction was exhibited by the varieties. The range of callus induction was varied from 62% to 74%. The highest callusing was found in CVE3 (74%) followed by (62.82). The highest percentage of shoot regeneration was found in CVE-3 (64.27 %) and the

lowest in Tri cap-1(50.33%). Different combinations and concentrations of BAP and IAA were used to observe callus induction and shoot regeneration. The highest percentage of callus induction (96%) and shoot regeneration (60.66%) was found in the combination of MS+2 mg/l BAP + 0.5 mg/l IAA. No callus induction and shoot regeneration was found without BAP and IAA. It was worth noting that percent shoot regeneration gradually increased with the increasing level of BAP upto 2 mg/l. further increasing of BAP level did not show any inprovement to shooting.

Two varieties of *C. capsularis* (vars. CVL1 and D154) were cultured in the presence of a range of pH level (e.g. 4.0, 4.5, 5.5, 6.0, 6.5, 7.0, 7.5) in association with MS plant regeneration medium.Varieties responded differently on various levels of pH.

Histochemical *GUS* assay was performed soon after co-cultivation of explants with the bacterium. *Agrobacterium tumefaciens* strain LBA 4404 contains the *GUS* gene. This gene produces blue colour with X-gluc through *GUS* histochemical assay. Therefore, the presence of this blue colour successfully confirmed the integration of *GUS* gene from bacterial into the plant cell.

Response of *GUS* assay was found to vary according to variety. Among the varieties, CVL1 showed the highest response (90% *GUS* positive) and showed the lowest response Tri cap-1 (80% *GUS* positive) to *GUS* assay.

Following infection and co-cultivation, the explants were cultured on selection and regeneration media containing kanamycin along with cefotaxime and growth regulators. *Agrobacterium tumefaciens* strain LABA4404 has *nptII* gene within its T-DNA, which confers kanamycin

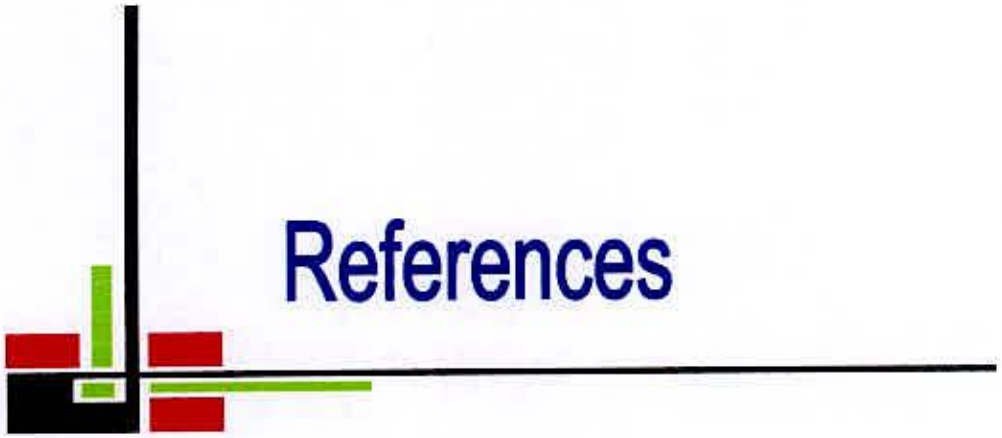
resistance of transformed cells. The putatative transformed regenerated plantlets grew on selection medium but died in course of time.

In the first experiment, an efficient and reproducible protocol for the regeneration of *Corchorus capsularis* genotypes has been developed using cotyledonary petioles as the explants.

As genetic engineering of crop plants relies on the development of efficient methods for the regeneration of viable shoots from cultured tissues, this protocol can be followed for genetic transformation of white jute.

MS medium supplemented with NaCl salt concentrations (25, 50, 75, 100 and 125 mM) was used for salt tolerance test of transformed plants. Transformed plants of two varieties were able to survive up to 100 mM salt concentration while the controlled plants died out in NaCl salt concentration of 50mM. The survival of transformed plants at 75 mM of NaCl salt concentration indicates the transformation of desired salt tolerant gene and successful expression of the gene in the varieties CVL-1 and Tri cap .It signals the possibility of the salt tolerant variety development using recombinant DNA technology for cultivation in the saline belt of Bangladesh.

An efficient protocol for the transformation of salt tolerant gene in white jute varieties has been developed in the second experiment, which showed the integration of two marker genes (*GUS* and *nptII*). Thus, in the future program agronomically important gene/genes can be transferred to the white genotypes using this protocol. Particularly, for the development of salinity resistant white jute varieties this technique of transformation can be exploited successfully.



References

REFERENCES

Ahmed, G., Hossain, A. B. M. M. and Islam, M. S. (1989). Regeneration of multiple shoots in jute *Corchorus olitorius* (var. O-4) from cotyledon and hypocotyls explants of germinating seeds. *Ind. J. Exp. Bio.* **27:** 334 -337.

Ahn, Y. K., Kim, H. V., Yoon, J. Y. and Park, H. G. 2001. Plant regeneration from leaf protoplasts of potato (*Solanum tuberosum* L.). *J. Korean Soc. Hort. Sci.,* **42(4):** 415-419.

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

Asano, Y., Otsuki, Y. and Meeusun, R. L. 1991. Insect control with genetically engineered crops. *Tibtech,* **9**: 197-200.

Baron, C., Losa M., Zhou S. and Zambryski P. C. 1997. VirB1, a component of the T-complex transfer machinery of *Agrobacterium tumefaciens* is processed to a C-terminal secreted product VirB1. *J. Bacteriol.,* **179**: 1203-1210.

BBS.(2003). Statistical Pocket Book of Bangladesh. Bangladesh Bureau of Statistics. Planning Division, Ministry of Planning. Govt.Peoples Republic of Bangladesh.

Bradley L.R., Kim J.S. and Matthysse A.G. 1997. Attachment of *Agrobacterium tumefaciens* to Carrot Cells and *Arabidopsis* wound sites

is correlated with the presence of a cell-associated, acidic polysaccharide. *J. Bacteriol.*, **179**: 5372-5379.

Binns, A.N. and Thomashow, M.F.(1988). Cell biology of *Agrobacterium* infection and transformation of plants. Annual Review of Microbiology **42**: 575-606.

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. *Biol. Sci.* **25**: 747-749.

Das, A. and Xie Y. H. 1986. Construction of transposon Tn3phoA: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. *Molecular Microbiology,* **27**: 405-414.

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., McCabe, 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.pp. 32.

Dodds, J. H. and Roberts, L. W.(1990).Anther and Pollen culture. In. J. It. Dodds and L.W. Roberts (Editors), Experiments in Plant Tissue Culture. Cambridge University Press, New York, NY, pp. 157-171.

Duan, X., Lix, Sue, Q., Abo-El-Saad, M., Xu, D. and Wu, R.(1996). Nat. *Biotechnol.* **14**: 494-498.

Ehsanpour, A. A. and Jones, M, G. R. 2000. Evaluation of direct shoot regeneration from stem cxplanls of potato (*Solanum tuberosum* L) cv. delware by thidiazuron TD7. *J. Sci. Tech. Agric. Nail. Res.*, **4(3)**: 47-54.

Evans, D. A. and Sharp, W. R. and Flick, C. E. (1981). Plant Tissue Culture: Methods and Applications in Agriculture. Academic Prees, New York, pp. 45-113.

Fiegert, A. K., Mix, W.G. and Vorlop, K. D. 2000. Regeneration of *Solanum tuberosum* L. cv. *Tomensa*: induction of somatic embryogenesis in liquid culture for the production of artificial seed. Landbauforschung Volkenrode, **50(3-4)**: 199-202.

Firoozabady, E., and D. L. DeBoer. 1993. Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). *In Vitro Cell. Dev. Biol.*, **29**: 166-173.

Fishhof, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Fraley, R.T.(1987). Insert tolerant transgenic tomato plants. *Biotechnol.* **5**: 805-807.

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

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

Ghosh, M; Saha,T; Nayak, P and Sen, K.(2002). Genetic transformation by particle bombardment of cultivated jute, *Corchorus capsularis* L. *Plant-Cell Rep*. **20**: 10, 936-942; 30 ref.

Gonggu, Z.(2000). Final Country Project Report-China. IJO proceedings of the final workshop on "Application of Biotechnology in the improvement of Jute, Kenaf and Allied Fibres- PhaseII." 10-12 Aug. 2000.Beijing, China, pp. 41-43.

Hamilton, C.M.(1997). A binary-BAC system for plant transformation with high-molecular-weight DNA. *Gene* **200**: 107-116.

Haseena, K., Zeba, I. S., Sarker, R. H., Khandekaer, L., Selina, B., Nazir, H., Mohammad, A., Hossain, M. B., Fatema, T. Z., Haque, S. and Shamim, H. (2000).

Herrera-Estrella, L.(1983). Transfer and _expression of foreign genes in plants. PhD thesis. Laboratory of Genetics, Gent University, Belgium.

Hilder, V.A., Gatehous, A.M.R., Sheerman, S.E., Barker, F.and Boulter, D. (1989). A novel mechanism of insect resis engineered into tobacco. Nature **330**: 160-163.

Hille, J., Wullems G., Schilperoort R. A. 1983. Non-oncogenic T-region mutants of *Agrobacterium tumefaciens* do transfer T-DNA into plant cells. *Plant Mol. Bio*. **2**: 155-163.

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

Hossain A.B.M.M., Ahmed, G., Khan, R.I.and Islam, M.S.(1998). Transient GUS expression in jute (*Corchorus capsularis* L. var D-154) explants after infection by *Agrobacterium 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.(1994). Genetic transformation of jute (*Corchorus capsularis* L. var. D-154): Gene delivery into jute cells by using *Agrobacterium tumefaciens*. Abstracts. 2nd Intl. Plant Tissue Culture Conference. December 10-12. Dhaka. p. 36.

Ibrahim, K. M., J. C. Collins, and H. A. Collins. 1990. Characterization of progeny of *Coleus blumei* following an *in vitro* selection for salt tolerance. *Plant Cell Tissue Organ. Cult.*, **28**: 139-145.

Islam, A.S. (1964). A rare hybrid combination through application of hormone embryo culture. Nature, 210, 310.

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., Haque, M. M. and Seraj, L.1.(1982). 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.

Jefferson, R. A.(1986). β-glucuronidase from *Escherichia coli* as gene fusion marker. *Proc. Natl. Aca. Sci. USA*. **86**: 8447-84451.

Jefferson, R. A., Kavanagh, T. A. and Bevan, M. W. (1987). GUS Fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO* ***J.*** **6**: 3901-3907.

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

Khatun, A., Davey, M. R., Power, M. R. and Cocking, E. C. (1990). Transformation of jute (*Corchorus spp.*) using *Agrobacterium rhizogenes* conferring a chimaeric neomycin phosphotranferase (*nptII*) gene. Poster proceedings of the International Congress on Plant Tissue and Cell Culture. Amsterdam, Netherlands, Abstract. pp. 2-82.

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. 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 Rep.* 1993, **13**: 1, 49-53.

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

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. 18th Bangladesh Science Conference,

Bangladesh Agricultural University, Mymensingh, Bangladesh, Abstract No. **66**.

Khatun, A.(1998). Biotechnological Approaches for Varietal Imporvement of Jute and Allied Fibres. Proc. of the Workshop on Application of Biotechnology in the Important of Jute, Kenaf and Allied Fibres – Phase I, IJO, Beijing, Chaina.

Khatun, A. (2001). Varietal improvement of jute through *Agrobacterium* mediated transformation. Final Report. Contract Research Project. Bangladesh Agriculture Research Council. pp. 4-17.

Khatun, A., Saha, C. K., Naher, Z., Mahbub, S., Siddique, S. and Bilkis, S. 2003. Plant regeneration from the cotyledons of tossa jute (*Corchorus olitorius* L.). *Biotechnol.* **3(3):** 206-213.

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* L.). *Biotechnol,* **2(2):** 86-93.

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

Larkin, P. J. and Scowcroft, W. R.(1982). Somaclonal variation: A new option for plant improvement. In: Vasil, 1. K., W. R. Scowcroft and K. J. Few (eds). Plant improvement and somatic cell genetics. New York. pp. 158-178.

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

Naher, Z., Khatun, A., Mahbub, M. A. Alim and Siddique, A. B. (2003). Influence of genotypes on plant regeneration from cotyledons of *C. capsularis* L. Biotechnol. **2(1)**: 44-51.

Naher, Z. and Khatun, A. (2004). Effect of different levels of pH on plant regeneration from cotyledons of *C. capsularis*. Annual Report. Bangladesh Jute Research Institute, Manik Mia Avenue, Dhaka. pp. 11-13.

Nehra, N.S., Chibbar, R.N., Kartha, K.K., Datta, R.S.S. and Crosley, W.L. (1990). Genetic transformation of strawberry by *Agrobacterium tumefaciens* using leaf disc regeneration system. *Plant Cell Rep.* **9**: 293-298.

Nester, E.W., Gordon, M.P., Amasino, R.M. and Yanofsky, M.F.(1984). Crown gall: a molecular and physiological analysis. Annual Review of Plant Physiology **35**: 387-413.

Pua, E., G. Sim, G. Chi, and L. Kong. 1996. Synergistic effect of ethylene inhibitors and putrescine on shoot regeneration from hypocotyl explants of Chinese radish (*Raphanus sativus* L) *in vitro*. *Plant Cell Rpt.* **15:** 685-690.

Purwati, R. D. and Sudarmadji, S. 1999. Response of five kenaf accession to shoot regeneration. *J. Penelit. Tanaman Ind. Indonesia*, **5(1)**: 31-37.

Rahman, S. M. Z., I-Iadiuzzaman, S., I laque, M.M. and Islam, A.S.(1985). Shoot Formation in *Corchorus capsularis* var. D-154 rrom Unorganized callus Bangladesh J. Bot. 14, 141-145. *with*

Agrobacterium rhizogenes and a binary vector plasmid. *Plant cell Rep.* **9**, 88-92.

Reichert, N. A., and D. Liu. 1994. Protoplast culture and in vitro regeneration of kenaf. Proc. Intl. Kenaf Assoc. Conf. **6**: 61-65.

Saha, M. N., Ghosh, M. and Sen, S. K. (1996). *In vitro* culture of jute (*corchorus capsularis* CVL-1) and its Transformation. M.Sc. Thesis, Dept of Biochemistry, University of Dhaka.

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

Schell, J., Van Montagu, M.(1997). The Ti-plasmid of *Agrobacterium tumefaciens*, a natural vector for the introduction of nif genes in plants. *Basic Life Sci.* **9**: 159-79.

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

Skirvin, R. M.(1978). Natural and induced variation in tissue culture. Euphytica, **27**: 241-266.

Smith, E. F. and Towsend C. O. 1907. A plant tumour of bacterial origin. *Science*, **25**: 671-673.

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

Thomas, J.C., Wasmann, C.C., Echt, C., Dunn, R.L., Bohneert, H.J. and McCoy, T.J. (1984). *Plant Cell Rep.* **14(1):** 33-37.

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

Vaeck, M., Reynaerts, A., Hofte, H. Jansens, S. and De, Beuckeleer, M. (1987). Transgenic plants protected from insect attack. Nature, London. **328(1):** 33-37.

Vierheiling, H., Altm, Neuhas, J.M., Boller, T.and Wiemken, A.(1993). *Molec. Plant Microbe Interaction.* **6:** 621-24.

Weising, K., Schell, J.andKhai, G.(1988). Foreign gene in plants: Transfer, Structure, expression and application. Annu. Rev. Genet. **22:** 421-477.

Wood, D. W., Setubal, J. C, Kaul, R. and Monks, D. E. 2001. The Genome of the Natural Genetic Engineer *Agrobacterium tumefaciens* C58. *Science,* **294**: 317-2323.

Zupan, J.R. and Zambryski, P.C. (1995). Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiol.* **107**: 1041.1047.

Appendices

APPENDICES

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

a) Macronutrients	**Concentration (mgL^{-1})**
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
b) Micronutrients	**Concentration (mgL^{-1})**
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
KI	0.83
Na_2MoO_4	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Iron source	**Concentration (mgL^{-1})**
$FeSo_4.7H_2O$	27.80
Na_2-EDTA.$2H_2O$	37.30
d) Organic solvent	**Concentration (mgL^{-1})**
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine acid	0.50
Thymine	0.10
Myo-inositol	100

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

Seed germination

Variable 3: No of seeds germinated

Grand Mean = 8.820 Grand Sum = 79.380 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.577	0.289	2.3971	0.2069
2	Factor A	2	3.108	1.554	12.9125	0.0180
-3	Error	4	0.481	0.120		
	Total	8	4.167			

Coefficient of Variation: 3.93%

s_ for means group 1: 0.2003 Number of Observations: 3
y

s_ for means group 2: 0.2003 Number of Observations: 3
y

Variable 4: **percent seed germination**

Grand Mean = 88.200 Grand Sum = 793.800 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	57.707	28.853	2.3971	0.2069
2	Factor A	2	310.847	155.423	12.9125	0.0180
-3	Error	4	48.147	12.037		
	Total	8	416.700			

Coefficient of Variation: 3.93%

s_ for means group 1: 2.0031 Number of Observations: 3

y

s_ for means group 2: 2.0031 Number of Observations: 3

Appendix III. Analysis of variance (mean squares) for no. of explants producing callus, Percent callus induction and Days required for shoot initiation

Variable 5: No. of EXPLANT SHOWING CALLUS

Grand Mean = 10.132 Grand Sum = 91.190 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	3.683	1.842	0.8399	
2	Factor A	2	4.914	2.457	1.1205	0.4108
-3	Error	4	8.772	2.193		
	Total	8	17.369			

Coefficient of Variation: 14.62%

s_ for means group 1: 0.8550 Number of Observations: 3
y

s_ for means group 2: 0.8550 Number of Observations: 3
y

Variable 6: Percent callus induction

Grand Mean = 67.420 Grand Sum = 606.780 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	161.122	80.561	0.8481	
2	Factor A	2	226.679	113.339	1.1932	0.3923
-3	Error	4	379.951	94.988		
	Total	8	767.752			

Coefficient of Variation: 14.46%

s_ for means group 1: 5.6269 Number of Observations: 3
y

s_ for means group 2: 5.6269 Number of Observations: 3
y

Variable 7: Days required for shoot initiation

Grand Mean = 10.000 Grand Sum = 90.000 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.000	0.000	0.0000	
2	Factor A	2	6.000	3.000	2.0000	0.2500
-3	Error	4	6.000	1.500		
	Total	8	12.000			

Coefficient of Variation: 12.25%

s_ for means group 1: 0.7071 Number of Observations: 3
y

s_ for means group 2: 0.7071 Number of Observations: 3
y

Appendix IV. Analysis of variance (mean squares) for Percent callus infection**, percent shoot regeneration and Average number of Shoot**

Variable 9: Percent callus infection

Grand Mean = 38.222 Grand Sum = 344.000 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	6.222	3.111	0.4375	
2	Factor A	2	150.889	75.444	10.6094	0.0252
-3	Error	4	28.444	7.111		
	Total	8	185.556			

Coefficient of Variation: 6.98%

s_ for means group 1: 1.5396 Number of Observations: 3
y

s_ for means group 2: 1.5396 Number of Observations: 3
y

Variable 10: Percent Shoot Regeneration

Grand Mean = 11.444 Grand Sum = 103.000 Total Count = 9

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	11.556	5.778	9.4545	0.0305
2	Factor A	2	48.222	24.111	39.4545	0.0023
-3	Error	4	2.444	0.611		
	Total	8	62.222			

Coefficient of Variation: 6.83%

s_ for means group 1: 0.4513 Number of Observations: 3
y

s_ for means group 2: 0.4513 Number of Observations: 3
y

Variable 12: **Percent shoot regeneration**

Grand Mean = 55.967 Grand Sum = 503.700 Total Count = 9

TABLE OF MEANS

1	2	12	Total
1	*	55.567	166.700
2	*	56.633	169.900
3	*	55.700	167.100
*	1	64.267	192.800
*	2	53.300	159.900
*	3	50.333	151.000

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	2.027	1.013	0.1834	
2	Factor A	2	323.207	161.603	29.2407	0.0041
-3	Error	4	22.107	5.527		
	Total	8	347.340			

Coefficient of Variation: 4.20%

s_ for means group 1: 1.3573 Number of Observations: 3
y

s_ for means group 2: 1.3573 Number of Observations: 3
y

Variable 11: Average number of Shoot

Grand Mean = 4.800 Grand Sum = 43.200 Total Count = 9

TABLE OF MEANS

1	2	11	Total
1	*	4.867	14.600
2	*	4.867	14.600
3	*	4.667	14.000
*	1	5.800	17.400
*	2	4.800	14.400
*	3	3.800	11.400

ANALYSIS OF VARIANCE TABLE

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.080	0.040	1.0000	
2	Factor A	2	6.000	3.000	75.0000	0.0007
-3	Error	4	0.160	0.040		
	Total	8	6.240			

Coefficient of Variation: 4.17%

s_ for means group 1: 0.1155 Number of Observations: 3
y

s_ for means group 2: 0.1155 Number of Observations: 3
y