Studies on *in vitro* plant regeneration and *Agrobacterium* mediated genetic transformation of jute - *Corchorus capsularis* L.

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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CERTIFICATE

This is to certify that the research work in the thesis titled "Studies on *in vitro* plant regeneration and *Agrobacterium* mediated genetic transformation of jute - *Corchorus capsularis* L." has been carried out by Ms. J. Pushyami Bharadwaj under my supervision for the full period prescribed under the Ph.D ordinance of this university and that this work has not been submitted for any other degree or diploma to any university or institute.

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DECLARATION

I hereby declare that the work presented in the thesis entitled "Studies on *in vitro* plant regeneration and *Agrobacterium* mediated genetic transformation of jute - *Corchorus capsularis* L." has been carried out by me under the supervision of Prof. P. B. Kirti, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad – 500 046, and that the work has not been submitted for any other degree or diploma to any university or institute.

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Dedicated to

My family

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CONTENTS

Abstract i
Abbreviations iv
Units vi
List of figures vii
List of tables ix

1. INTRODUCTION
1.1 Introduction 1
1.2 Scope of the present study 11
1.3 Objectives 12

2. LITERATURE REVIEW
2.1 Jute: An important fiber crop 13
2.2 Taxonomy and general characteristics of jute 13
2.3 Advantages of *C. capsularis* over *C. olitorius* 14
2.4 Major diseases and pests 15
2.5 Genetic improvement by conventional breeding in jute 15
2.6 Jute tissue culture 16
2.7 Gene transfer methods 20
2.7.1 Direct gene transfer/ Particle bombardment 20
2.7.2 *Agrobacterium* mediated/ vector mediated 21
2.8 Identification of transgenics 23
2.8.1 Scorable reporter 23
2.8.2 Selectable marker 24
2.9 Progress in jute protoplast studies 25

3. ESTABLISHMENT OF AN EFFICIENT REGENERATION SYSTEM IN AN INDIAN VARIETY OF WHITE JUTE
3.1 INTRODUCTION 26
3.2 MATERIALS AND METHODS
3.2.1 Seed surface sterilization and seed germination 28
3.2.2 Identification of a responsive explant from various cultivars of jute (*C. capsularis* L.) 28

3.2.3 Chemicals 28
3.2.4 Stock Solutions 28
3.2.5 Culture medium 29
3.2.6 Preparation of explants from aseptically grown seedlings 29
3.2.7 *In vitro* plant regeneration from seedling derived cotyledonary petiole explants
3.2.7.1 *In vitro* regeneration experiments 30
3.2.7.2 Establishment of regenerated plants 30
3.2.8 Histological examination 31
3.2.9 Optimization of the age of seedlings 31
3.2.10 Data collection and statistical analyses 31
3.3 RESULTS
3.3.1 Comparison of response of different genotypes in tissue culture 32
3.3.2 Effect of plant growth regulators on different explants of cultivar JRC 321
3.3.2.1 Hypocotyls as source of explants 33
3.3.2.2 Cotyledons as source of explants 34
3.3.2.3 Mature embryo as explants 36
3.3.3 Response of explants towards treatment with plant growth regulators for *in vitro* plant regeneration
3.3.3.1 Shoot regeneration 37
3.3.3.2 Histological analysis 40
3.3.3.3 Effect of vitamins on multiple regeneration of cotyledonary petioles 41
3.3.3.4 Effect of age of seedling 42
3.3.3.5 Shoot elongation and rhizogenesis 44
3.4 DISCUSSION 45
3.5 CONCLUSION 47

4. STUDIES ON *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION OF INDIAN VARIETY OF WHITE JUTE
4.1 INTRODUCTION 48
4.2 MATERIALS AND METHODS
4.2.1 Marker genes for plant transformation 50
4.2.1.1 Scorable reporter genes 50

4.2.1.2 Selectable marker genes 51
4.2.2 Preparation of competent cells of *Agrobacterium* 52
4.2.3 Transformation of *Agrobacterium* cells 52
4.2.4 Plasmid isolation from *Agrobacterium* 52
4.2.5 Optimisation of parameters for regeneration amenable transformation 53
4.2.6 Histochemical assay of *GUS* 53
4.2.7 *Agrobacterium* mediated transformation of cotyledonary petioles
4.2.7.1 *Agrobacterium* culture preparation for transformation 54
4.2.7.2 Transformation procedure 54
4.2.8 DNA extraction from leaf tissues 55
4.2.8.1 Purification of genomic DNA 56
4.2.8.2 DNA Precipitation 56
4.2.8.3 Quantification of DNA 57
4.2.9 *In planta* genetic transformation of seedlings by *Agrobacterium* infection of apical meristems 57
4.2.10 Molecular analysis using PCR amplification of *nptII* and TvD_1 gene 58
4.2.11 *In planta* genetic transformation of mature embryos by *Agrobacterium* carrying p35S*GUS*INT vector 59
4.2.11.1 GUS histochemical assay of mature embryos 59
4.3 RESULTS AND DISCUSSION
4.3.1 Marker genes for plant transformation 60
4.3.1.1 Response of explants on kanamycin as plant selectable marker 61
4.3.1.2 Response of leaf discs on gluphosinate as plant selectable marker 62
4.3.2 Response of explants towards *Agrobacterium* infection upon various parameters based on *GUS* assay 63
4.3.2.1 Effect on addition of acetosyringone 64
4.3.2.2 Use of different bacterial strains and effect of bacterial concentration 66
4.3.2.3 Effect of vacuum infiltration on transformation 67
4.3.3 Transient GUS expression 67
4.3.4 Evaluation of putative transgenic plants obtained from

transformation of cotyledonary petioles 67
4.3.5 Evaluation of putative transgenic plants obtained from transformation of apical meristems 69
4.3.6 Stable *GUS* expression observed in various stages of transformed mature embryo explants 71
4.4 CONCLUSION 73

5. STUDIES ON REGENERATION AND PROTOPLAST CULTURE OF HYPOCOTYL EXPLANTS IN JUTE, *CORCHORUS CAPSULARIS L.*
5.1 INTRODUCTION 74
5.2 MATERIALS AND METHODS
5.2.1 Source of explants 76
5.2.2 Regeneration experiments
5.2.2.1 Culture media and culture condition 76
5.2.2.2 Rooting and hardening 77
5.2.3 Protoplast isolation 77
5.2.3.1 Purification of the protoplasts 77
5.2.3.2 Protoplast Culture 78
5.3 RESULTS AND DISCUSSION
5.3.1 Regeneration experiments 80
5.3.1.1 Rooting and hardening..... 81
5.3.2 Protoplast isolation 82
5.3.2.1 Protoplast culture 84
5.4 CONCLUSION 87

6. CONCLUDING REMARKS
6.1 Significance and salient features of the study 88
6.2 Future prospects 90

7. BIBLIOGRAPHY 91

8. LIST OF PUBLICATIONS 104

Abstract

Abstract

Jute (*Corchorus sp.*) is an important fiber crop cultivated for its bast fibers. The fiber is a commercial commodity next to cotton and generates revenue to the country. The cultivated species *C. capsularis* and *C. olitorius* are grown for the fiber in India, Bangladesh, and China etc. The largest producers being India and Bangladesh. The crop is gaining commercial importance due to its value added products produced from its fibers that are biodegradable. Farmers are looking forward for varieties that yield good quality and quantity of fibers. There are major threats to the crop from biotic and abiotic factors due to which there is lot of damage to the crop. Crop improvement thus needs the introduction of specific genes for specific purpose. Genetic diversity in these two species has been narrowed down due to restricted selection from natural populations for quantitative traits. In addition, a strong sexual incompatibility, which causes premature cessation of embryo growth, exists between these two preventing any cross breeding (Kundu et al., 1956; Ganesan et al., 1957). Hence, creating inter-specific hybrids via natural or conventional breeding is highly impossible. For the same reason, possibility of improving the fiber has become almost impossible. The only possible way for crop improvement is through biotechnological intervention. In jute, absence of an efficient system for shoot multiplication and plant regeneration amenable for *Agrobacterium* mediated transformation, mechanisms for increasing the T-DNA transfer to regenerating cells and strategy for identifying transformed and untransformed cells have been identified as major bottlenecks for implementing transgenic approaches.

Significantly higher shoot regeneration (67.8 %) was obtained from seedling derived cotyledonary petiole explants. The explants were further used in *Agrobacterium* mediated transformation experiments. To discriminate between the transformed and non-transformed shoots, some of the pre-requisite experiments and factors were standardized to achieve the maximum number of shoots after transformation and co-cultivation. Kanamycin and phosphinothricin selection system were established using cotyledonary petiole explants and leaf discs respectively. Before proceeding for transformation experiments, optimal conditions

for transformation based on transient GUS activity that represents early infection were identified using *Agrobacterium* strain harboring binary vector p35S*GUS* INT. The highest infection frequency of cotyledonary petioles was obtained with bacterial strains, EHA105 (OD 0.3) or LBA4404 (OD 0.5) by subjecting the tissues to vacuum and supplemention with 100 µM Acetosyringone. These results establish that *A. tumefaciens* can be employed for stable genetic transformation in cotyledonary petioles of white jute, since plant regeneration from this species is successful.

Although fertile transgenic plants that were confirmed by PCR analysis could be obtained in the T_0 generations, the efforts to repeat this in the subsequent generations did not succeed. The failure of the gene to get transmitted to subsequent generations might be attributed to the genetic instability or the impotency of the plants to segregate the gene in the consecutive generations. We have encountered the same failure when alternate approaches of *in planta* method of genetic transformation were applied. Mature embryos when infected with *Agrobacterium* strain harboring binary vector p35S*GUS* INT showed blue coloration in the embryos as well as germinated seedlings. Mature embryos might be a potential source of explants for transformation in this crop species.

Generation of hybrids is almost impossible through conventional breeding practices due to the presence of strong sexual incompatibility barrier. Hence exploring the protoplast fusion technique offers a viable option. Till date, protoplast research in jute is not successful due to the constraints in isolation and culture techniques. In our experiments, we could isolate good quality and quantity of protoplasts from hypocotyls using enzyme digestion method and could easily eliminate the problem of mucilage by altering the temperature and growth conditions of the seedlings. Dividing protoplasts could be obtained which developed into microcalli. However, these microcalli turned brown and soon necrosis was observed in subsequent cultures.

In our present study, we could establish an efficient and reproducible regeneration system in the local cultivar of deshi jute using cotyledonary petiole

explants. Parameters for *Agrobacterium* infection were optimized. However, our attempts to transform this recalcitrant crop did not meet with success. Protoplast isolation and culture technique were well established irrespective of the variety and cultures could be maintained till microcalli stage. But the culture conditions for transforming microcalli to macrocalli for further regeneration need to be standardized further.

Abbreviations & Units

Abbreviations

2,4–D	2,4-Dichlorophenoxyacetic acid
β- Merc	β-Mercapto ethanol
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
B_5	Gamborg's Medium (1968)
BAP	6-Benzylaminopurine
CAMBIA	Centre for Application of Molecular Biology to International Agriculture
CaMV	Cauliflower Mosaic Virus
cDNA	Complementary DNA
CTAB	Cetyl/ Hexadecyltrimethyl Ammonium Bromide
DDW	Double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
dsDNA	Double stranded DNA
EDTA	Ethylene Diamine tetra acetic acid
EtBr	Ethidium Bromide
GA_3	Gibberlic acid
GUS	*β- 1- 4 glucuronidase*
HCl	Hydrochloric acid
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid

IPTG	Isopropyl-β-D thiogalactoside
KN	Kinetin
LB	Luria Bertani
MS	Murashige & Skoog (1962)
NAA	Naphthalene Acetic Acid
NaOH	Sodium Hydroxide
nptII	*neomycin phosphotransferase II*
OD	Optical Density
PCR	Polymerase Chain Reaction
PVPP	Polyvinyl Polypyrrolidone
RT	Room Temperature
RPM	Revolutions per minute
SDS	Sodium Dodecyl Sulfate
ssDNA	Single stranded DNA
TAE	Tris acetic acid EDTA
T-DNA	Transferred DNA
TDZ	Thidiazuran/ 1-phenyl- 3- (1,2,3 – thiadiazol - 5 - yl) urea
TE	Tris EDTA
Ti- Plasmid	Tumor inducing plasmid
Tris	Tris (hydroxyl methyl) amino methane
uidA	β- glucuronidase gene
vir	*virulence gene*
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide

Units

µg	microgram
µl	microliter
µM	micromolar
^{o}C	degree celsius
bp	base pair
cm	centimeter
d	day
Da	daltons
g	gram
h	hour
kb	kilobases
kDa	kilo Dalton
M	molar
mg	milligram
mgl^{-1}	milligram per liter
min	minutes
ml	milliliter
ng	nanogram
nm	nanometre
pH	negative log of H+ ion
psi	pounds per square inch
pmol	picomol
rpm	revolutions per minute
s	second

Tables & Figures

List of figures

Figure No.	Title	Page No.
1	Diversification of jute	5
2	Approaches for crop improvement in jute	6
3	Response of hypocotyl cultures on BAP and IAA containing medium	33
4	Response of cotyledon explants on NAA and BAP containing medium	34
5	Embryos cultured on TDZ containing medium	36
6	Plant regeneration by organogenesis from cotyledonary petiole explants in Jute (*Corchorus capsularis* L.*)* cv. JRC 321	38
7	Histological analysis of emergence of shoot buds at cotyledonary petiole	40
8	Graph illustrating the effect of age of seedling with the mean number of explants producing shoots	42
9	Action of β- glucuronidase on X-Gluc	51
10	Partial map of vector pCAMBIA 2300 harboring TvD_1 gene cassette	57
11	Effect of kanamycin on cotyledonary petioles	61
12	Comparison between Basta® concentrations on the control leaves	62
13	β-glucuronidase (GUS) transient assay of cotyledonary petioles of jute	63

Figure No.	Title	Page No.
14	Graph illustrating effect of various parameters affecting transformation efficiency by transient GUS assay	65
15	PCR for the T_0 putative transgenic plants showing the amplification of 1kb *nptII* marker gene	68
16	PCR for the T_0 putative transgenic plants showing the amplification of 570kb *TvD_1* gene	70
17	Pod size between control and the putative transformed plant	70
18	GUS localization in mature embryo explants of jute	72
19	In vitro plant regeneration from hypocotyl segments of jute (*Corchorus capsularis* L.) cv. JRC 698	82
20	Protoplasts isolated from hypocotyls of Jute (*Corchorus capsularis* L.*)* cv. JRC 698	86

List of tables

Table No.	Title	Page No.
1	Comparison of nutritional value between the leaves of spinach and jute	3
2	Characters of the *capsularis* varieties of jute	14
3	Status of *In vitro* regeneration in *C. capsularis* L.	18
4	Status of transformation in *C. capsularis* L.	22
5	Effect of growth regulators on cotyledon explants of jute (*C.capsularis* cv. JRC 321)	35
6	Effect of IAA, NAA, 2,4 D and BAP on hypocotyls and cotyledonary petiole explants of Jute (*C. capsularis* cv. JRC 321) in shoot bud induction after four weeks of incubation	39
7	Influence of vitamins on shoot regeneration	41
8	Multiple comparison (*F*-statistics of shoot bud induction)	43
9	Results of Jute transformation using cotyledonary petioles as explants	68
10	Solutions and culture medium used for protoplast isolation and culture	79
11	Percentage shoot bud induction in hypocotyl explants in response to 6- BAP and adenine hemisulfate supplemented medium	81

Chapter 1

CHAPTER 1

1.1 Introduction

Jute is a fiber crop producing soft, shiny, long, off-white to brown, and cheapest available natural vegetable fiber that could be spun into strong threads. Though its use dates back to 800 BC by the use of its leaves in ancient medicine, the importance and wide acceptance for use in sophisticated textile products and in fashion industry occurred only in the nineteenth century. Of the more than 100 species of the genus *Corchorus*, only two, *C. capsularis* and *C. olitorius* are cultivated for fiber production. The two species – *C. capsularis* (white jute) and *C. olitorius* (tossa jute) yield a cellulosic bast fiber, which is the second largest traded commodity after cotton (Kundu et al., 1956). Due to some of its good spinning characteristics, it is well known as "*golden fiber*".

Cultivation

Favorable conditions for jute cultivation are found in the deltas of the great rivers of their tropics and sub-tropics- the Ganges, the Irrawaddy, the Amazon, and the Yangtze, where irrigation, often by extensive flooding, and alluvial soils combined with long day lengths prevail. The crop however thrives very well under rain-fed and hot humid and sub-tropical conditions in the Bengal basin in India and Bangladesh where more than 80 % of the crop of the world is grown. India with overall of ~58% of worlds production tops the production of jute. Bangladesh with ~33% lies at second position followed by China with ~3%.

Cultivation – climate and soils

The cultivation of jute in India is mainly confined to the eastern region states - West Bengal, Bihar, Assam, Tripura, Meghalaya, Orissa and Uttar Pradesh. Nearly 50 percent of total raw jute production in India alone figures in West Bengal. In India 4000,000 families are involved in the cultivation of raw jute. Jute is a rain fed crop – sown during March till May and harvested during June to September. It requires a warm and humid climate with temperature between 24° C to 37° C and relative

humidity of 70% – 80% for successful cultivation. Jute is harvested any time between 120 days to 150 days when the flowers have been shed. Early harvesting gives good healthy fibers.

Jute leaves in medicine

While better known as a fiber crop, jute is also a medicinal vegetable. This vegetable is popularly known as Saluyot. Jute leaves contains protein, calories, fibres as well as antitumor promoters- phytol and monogalactosyl-diacylglycerol. It may reduce the risk of cancer. *Capsularis* (deshi) has maximum use as vegetable than *olitorius* (tossa) due to its bitter taste. The dwarf mutant line CM-18 was selected after induced mutation of its parent variety CVL-1 for higher leaf yield. Due to better performance in respect of yield and quality the mutant CM-18 has been registered as the first jute variety in Bangladesh for vegetable purpose in the name of *Binapatshak-1* in 2003. The mutant CM-18 has high vegetable yield potential, protein and carotene content. Low alkaloid contained in leaves make it tasty (Shamsuzzaman et al., 2003).

In addition to adding a distinct flavor to food, jute leaves also have nutritional value. Like spinach and other green leafy vegetables, its leaves can be cooked or chopped for use as vegetable. A comparison of the nutritional requirements provided by jute and spinach affirms the importance of this crop in human nutrition and health care.

Table 1: Comparison of nutritional value between the leaves of spinach and jute

Ingredients and unit	Spinach leaf	Jute leaf
Calories (k cal)	25	73
Protein (g)	3.3	3.6
Lipid (g)	0.2	0.6
Calcium (mg)	55	298
Iron (mg)	3.9	11
Carotene (mg)	5200	6400
Vitamin B-1 (mg)	0.13	-----
Vitamin B-2 (mg)	0.23	-----
Vitamin C (mg)	65	64

Jute leaves are also used as demulcent, deobstruent, diuretic, lactagogue, purgative, and tonic. It is a folk remedy for aches and pains, dysentery, enteritis, fever, dysentery, pectoral pains, and tumors (Duke et al., 1983).

Diversification of jute products

The fiber is mainly used for the manufacturing of traditional products such as hessian cloth, food grade bags, carpet backing and floor covering. Diversified jute products include geo-textiles for land erosion control, jute-reinforced plastics, jute laminates, pulp and paper, decorative fabrics, carpets and handicrafts. The traditional excellence of Indian artisans is well reflected in the jute handicrafts of utility, decoration and novelty. Its UV protection, sound and heat insulation, low thermal conduction and anti-static properties make it a wise choice in home décor. Also, fabrics made of jute fibers are carbon- dioxide neutral and naturally decomposable. Jute non-wovens and composites can be used for underlay, linoleum substrate, and more.

Rightfully placed as eco-friendly natural fiber with versatile applications, prospects range from low value geotextiles to celluloid products (films), non-woven textiles and composites. Geotextiles are more popular in the agricultural sector. It is a lightly woven fabric made of natural fibers that is used for soil erosion control, seed protection, weed control, and many other agricultural and landscaping uses. It can be used more than a year and being bio-degradable keeps the ground cool and is able to make the land more fertile when left to rot in the ground.

Future of raw jute fiber lies mainly in quality improvement of fibers for producing diversified and value-added uses. The world market for this crop is negatively influenced with the stiff competition by the synthetic fibers and positively by the market expansion for the diversified products of jute. Factors such as heavy weight and the related unsuitability of jute sacks for automatic filling systems as compared to polypropylene sacks; the superior breathability of jute sacks and biodegradability of jute vis-à-vis the water-resistant qualities of synthetic sacks have resulted in the contraction of jute market. Being an annual crop subject to instability due to weather, reliable and regular supplies of fiber of consistent quality have resulted in further displacement of jute by synthetic fibers. However, there are some factors, such as the preference for jute packaging for food grains due to its breathability, as well as jute sacks re-usability that may work to offset the impact of the above issues.

Supported by several technological developments today, jute can be used to replace expensive fibers and scare forest materials. The Indian Jute Industries Research Association (IJIRA) in association with Indian Jute Industry has recently developed hydrocarbon free jute bags that are of food grade quality. These bags are used for packaging food stuffs and have great demand throughout the world.

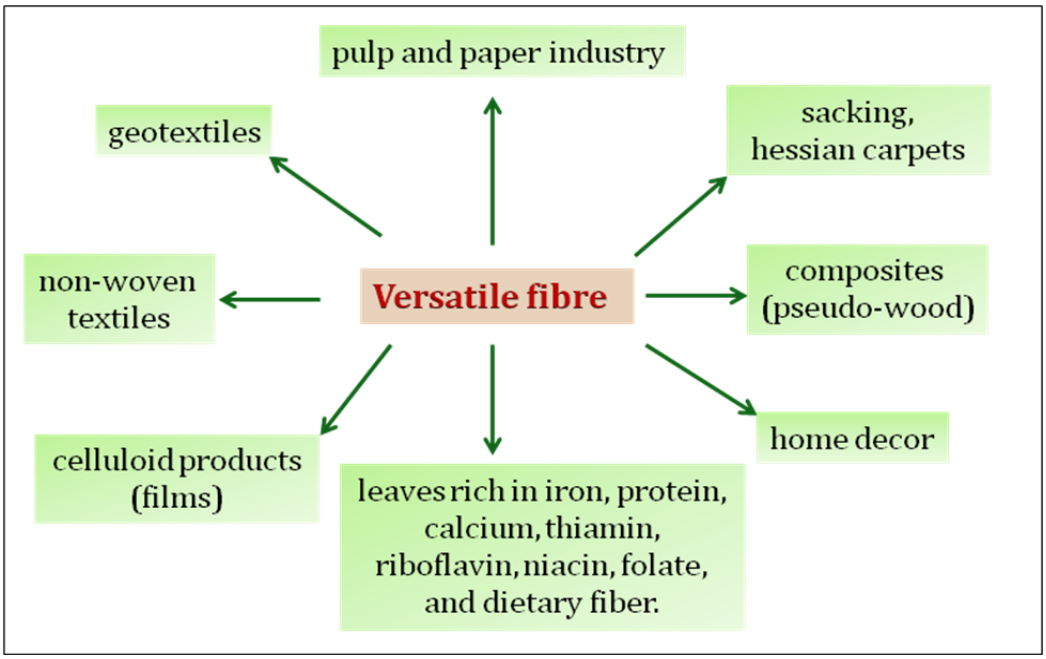


Fig. 1: Diversification of Jute

Jute quality is judged in terms of strength, fineness and lignin content of the fiber. The principle uses of jute yarn are for industrial purposes in which adequate strength is normally regarded as essential. It is therefore regarded that the yarn should be of finer counts (6 lb/ spy and below). Also, such yarn produced should be converted to light, dense and strong hessian fabrics for ready acceptability in the market. From plant anatomical view fiber quality depends on the length-breadth ratio of ultimate fiber cells. Higher the ratio finer is the fiber. (Sen et al., 2009 Int. Con.). Some of the wild species of jute have finer fiber quality. High lignin content in these fibers is responsible for color instability of the dyed jute products. Reducing lignin content to the optimum level by genetic manipulation offers a solution. In view of this, production of high yielding and better quality jute varieties need attention for improving the present condition of this natural fiber crop. Through appropriate breeding practices, these characters can be exploited in the cultivated species. Conventional breeding practices have proved to be of limited success due to their narrow genetic base and presence of strong sexual incompatibility barrier between the two species (Patel and Datta 1960; Islam and Rashid 1960; Swaminathan et al., 1961; Sarker and Hoque 1994). Thus, alternate methods of

somatic hybridization or gene manipulation have to be adopted. This can be achieved in three broad ways, viz.

(i) Conventional breeding technique – refers to selection of superior genotypes evolving from genetically variable populations derived from sexual recombination. This method has its own limitations in this crop due to incompatibility barrier between the species.

(ii) Genetic engineering – process of transgenesis – genes from unrelated species can be transferred for crop improvement. Genetic manipulation is a very important approach to achieve the targeted quality improvement.

(iii) Protoplast fusion - an important tool in crop improvement for bringing genetic recombinations and developing hybrids.

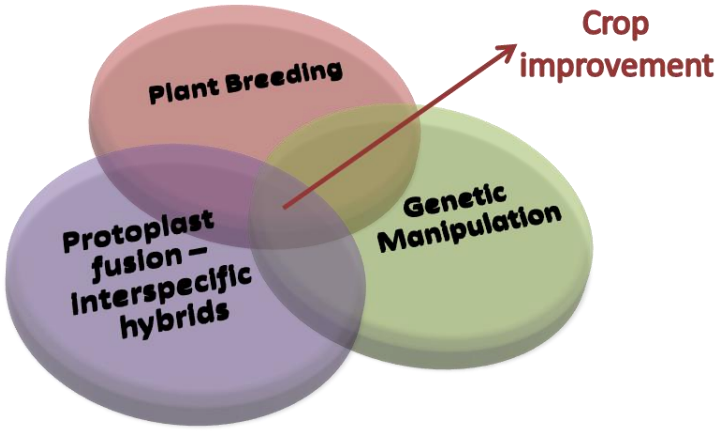


Fig. 2: Approaches for crop improvement in jute

Culture of jute tissues *in vitro*

Attempts have been made to regenerate this crop by several researchers (Rahman et al., 1985; Seraj et al., 1992; Abbas et al., 1997; Saha et al., 1999; Naher et al., 2003; Sarker et al., 2007). The regeneration protocols are difficult to reproduce (Sarker et al., 2007) and regeneration is genotype dependent (Naher et al., 2003). Most of the reported protocols used the local cultivars of Bangladesh except for one report by Saha and Sen (1999) that reported regeneration of an Indian variety using cotyledon explants cultured in liquid medium. However, their protocol failed to

develop regenerated plantlets. Development of an efficient plant regeneration system amenable to genetic transformation is the bottleneck in developing transgenics in jute, a highly recalcitrant cash crop.

Genetic transformation

Agrobacterium is a ubiquitous member of soil microflora, most of which are saprophytic in nature and the soil bacterium cause neoplastic diseases in dicots. The use of *Agrobacterium* as the tool for genetic transformation has greatly increased in recent years. Pathogenecity of this species was discovered three decades ago that it is a specialized process involving transfer of genes between the bacterium to the plant and involves several factors (Chilton et al., 1977). Upon infection, *A.tumefaciens* is capable of transferring the T-DNA, a DNA segment of Ti plasmid into the plant cell that gets integrated into the genome (Binns and Thomashaw, 1988) and is stably expressed. Any foreign DNA placed between the T-DNA borders can be transferred, integrated and expressed in the plant cell. Many reviews on plant transformation mediated by *A. tumefaciens* have shown that the transfer and integration of T-DNA into plant genome is influenced by several plant and *A.tumefaciens* specific factors like bacterial strain, plant genotype, *vir*-gene inducing synthetic phenolics compounds, explant type, mode of infection etc. Since all these factors have a synergistic role in the transfer, integration and stable expression of foreign genes in the host plant genome, it is worth considering each of them during the process of plant transformation.

Advantages of *Agrobacterium* mediated gene transfer

Agrobacterium mediated genetic transformation overcomes the conventional breeding methods with the introduction of genes for desired traits directly into the host plant genome. The significant advantages of using *Agrobacterium*-mediated transformation over other transformation methods are: wide host range of the *Agrobacterium* i.e can infect most of the plants with very few exceptions, integration of small copy number of transgene into host genome and stable integration of the transgene (newly introduced gene) into the plant genome. However, it is difficult to

get consistent results using this method of transformation as this method involves several factors- both bacterial and host which need to be thoroughly optimized.

Genetic transformation of jute tissues

An efficient transformation system would provide avenues to introduce genes for agronomic characters in this crop species for crop improvement. The essential requirements in a gene transfer system for production of transgenic plants are:

(i) Reliable and reproducible method for plant regeneration from somatic tissues.

(ii) A method for gene transfer, integration and expression.

(iii) A system to differentiate the transformed and the non-transformed cells (selectable marker).

Gene transfer by particle bombardment has been attempted in an Indian cultivar of jute (Ghosh et al., 2002). However, the procedure is still far from routine for large scale recovery of transgenics with desirable traits. Sarker et al., (2008) established a method for genetically transforming this crop only in the local varieties of Bangladesh. They observed that none of the plantlets obtained from petiole attached cotyledon explants after multiple regeneration survived in the presence of kanamycin (100 mg l^{-1}) in the subsequent stages. Jute transformation still remains inefficient and production of transgenics remains tough due to genotype dependency and poor transformation efficiency. Till date, there are no attempts of *Agrobacterium* mediated genetic transformation in the local Indian species of jute. Therefore, attempts were made to transform this crop by *Agrobacterium* infection of the tissues that regenerated whole plants.

For identifying or selecting those cells that have integrated the foreign DNA by agro-infection, selectable marker genes have been pivotal to the development of plant transformation technologies, because the marker genes allow scientists to identify the cells that express the cloned DNA. Resistance to kanamycin and phosphinothricin, which were most commonly used selectable markers, are to be standardized for their minimal inhibitory concentration.

Considering the recalcitrant nature of the target crop towards regeneration of the transformed tissues, alternate methods of transformation have to be opted for. *In planta* transformation method overcomes the disadvantages of the conventional *in vitro Agrobacterium* mediated transformation method. *In planta* transformation does not involve *in vitro* culture of plant cells or tissue, which is its greatest advantage.

Protoplast cultures in jute

The merit of this natural fiber over the synthetic fibers necessitates the intervention of biotechnological efforts for crop improvement. Major impediment in jute improvement is the strong sexual incompatibility that exists between the two cultivated species of jute. Though there are reports of interspecific hybrids in the past, the fact remains that they could not be established in the field and the hybrids did not exhibit the recombinant nature. Any alternate technique to transfer genes of interest opens scope for cultivar improvement in this crop. One such technique, used successfully in other crops to overcome sexual incompatibility, is protoplast fusion.

Protoplast fusion requires the development of a regeneration system from protoplasts. Furthermore, an efficient and reliable regeneration system from protoplasts opens up new opportunities for genetic transformation via direct DNA uptake or electroporation, as in many other crops. Somatic hybridization and genetic modification may enhance the genetic variability and one could aim at improving them by employing these techniques. Until now, there was no report on plant regeneration from protoplasts in *Corchorus capsularis* for reasons that the jute tissues and cells in culture have long been extremely recalcitrant (Saha & Sen 1992; Naher et al. 2003). Somatic embryogenesis was reported from the mesophyll and hypocotyl derived protoplasts, but these embryos did not develop further into plantlets (Saha and Sen, 1992). Saha and Sen (2001) presented a technique of protoplast culture from cotyledons, but they could not develop into microcalli, which was a rare event. A method to routinely isolate protoplasts in large quantity

and to regenerate them on solid medium would provide new avenues for crop improvement in this species.

The present study was undertaken with the objectives to establish an efficient plant regeneration system that could be further used for *Agrobacterium* mediated genetic transformation, formulate a selection system for kanamycin and phosphinothricin as selectable markers, establish alternate methods of T-DNA delivery such as *in planta* methods using apical meristems of young seedlings and mature embryos and finally study the possibility of establishing an efficient method for protoplast isolation and culture.

1.2 Scope of the present study

Improvement of existing cultivars with the application of biotechnological techniques is needed for jute. Due to lack of useful genes in the existing cultivars and due to genetic incompatibility barrier, crop improvement in this species stands an issue. Genetic modification of elite genotypes can be used to transfer useful genes within a short period of time. To establish these techniques in jute, an efficient regeneration protocol and transformation system must be developed. With these systems in place, transgenic jute plants could be obtained. Therefore three objectives were studied and will be discussed here.

The first chapter deals with the importance of the crop and its diversification in the market. In chapter 2, a brief summary of the past findings in this crop was presented.

Chapter 3 describes a method for efficient regeneration of explants in an Indian species of white jute that could be combined with transformation.

In chapter 4, *Agrobacterium*-mediated transformation using cotyledonary petioles is described and optimized by an investigation into several parameters such as the bacterial strain, concentration of bacterial suspension, use of compounds to induce virulence and the method of co-cultivation. The optimized parameters have been used for transforming this crop. Alternate methods for genetic transformation have been discussed.

Chapter 5 describes for the first time in jute a regeneration system using hypocotyl explants and protoplast culture in jute. Parameters optimized for protoplast isolation and culture methods are also discussed.

Finally, the importance of the present work and future prospects are discussed in Chapter 6.

1.3 Objectives

The present investigation was carried out with broad objectives to establish efficient plant regeneration from seedling derived explants that could be used further in crop improvement. These regenerable explants were tried with *Agrobacterium* infection for genetic transformation of the crop. Transgenic plant selection systems for kanamycin and phosphinothricin (basta®) were optimized for their respective minimal inhibitory concentrations on the explants. Hypocotyl explants were used for isolation and culture of protoplasts for calli development. The results obtained are discussed in the three chapters below:

1. Establishment of an efficient regeneration system in an Indian variety of white jute
2. Studies on *Agrobacterium* mediated genetic transformation of white jute of Indian cultivar
3. Studies on regeneration and protoplast culture of hypocotyl explants in jute, *Corchorus capsularis* L.

Chapter 2

CHAPTER 2

2.1 Jute: an important fiber crop

On a global scale, jute is the second most important cultivated fiber crop after cotton (Kundu et al., 1956) and also in the Indian subcontinent (Basu et al., 2004). The "*golden fiber*" as well known to the world, is used for many commercial and domestic applications (Mia et al., 1967). Indo-Bangladesh subcontinent accounts for more than 90% of the total world productions of the raw jute fibers (Hossain et al., 1994). The crop has considerable commercial significance for the generation of diversified value-added industrial products, in addition to its potential for industrial production of packaging material. As natural fiber, it possesses both the qualities of renewability and biodegradability. It can thus be called as 'fiber of the future".

2.2 Taxonomy and general characteristics of jute (Banerjee et al., 1955)

The genus, *Corchorus*, belongs to the family *Malvaceae*, formerly *Tileaceae* (Barbara et al., 2003). Though there are over 100 species of *Corchorus*, only two, *Corchorus capsularis* L. and *C. olitorius* L., are cultivated widely (Sarker and Al-Amin 2007). The origin of white jute is said to be Indo-Burma including South China, and that of tossa jute to be Africa (Kundu et al., 1951). It includes about 40 species mostly distributed in the tropical regions. Fourteen of them including *C. capsularis* and *C. olitorius* are diploid (2n=14) and four are tetraploid (2n=28). *C. capsularis* and *C. olitorius* are both herbaceous annuals. The vegetative period of both is about 3-5 months. At the harvest stage, varieties of *C. capsularis* attain a height of about 5-12 feet and those of *C. olitorius* 5-15 feet or more. The stems of both are cylindrical. Leaves of both species four to five inches long and are glabrous, bright green acuminate and serrated. Flowers of both the species look yellow, are small in size and occur in condensed cymes occuring in the axils of leaves, and composed of 4-5 sepals and petals, 5 to numerous stamens. Ovary is 2-6 locular. Seeds are small and numerous. Seeds of *C. olitorius* are smaller. *C. capsularis* seed is coppery in color and weigh about 500-600 seed per 1 gram. *C. olitorius* seed is greyish in color and weigh about 1000 seeds per 1 gram. Both the species are mostly self pollinated.

2.3 Advantages of *C. capsularis* over *C. olitorius*

C. capsularis and *C. olitorius* (tossa jute) have their own unique qualities. While *C. capsularis* could adapt itself to varied growing conditions and has better tolerance to water logging, *C. olitorius* possesses better yield, resistance to pests and good retting quality (Finlow et al., 1939). About 75% of the total acreage cultivation is dominated by *C. capsularis.* It can be grown on low and high lands, and can withstand water logging and hence confined to West Bengal region of India due to its topography of land. *C. olitorius* grows well on high lands and hence, grown mostly in Bangladesh (Banerjee et al., 1955). Earlier major emphasis was on the evolution of high yielding varieties only. Due to increased trade demand for value added products, development of improved high yielding varieties with improved fiber quality and suitability for diversified uses is also a major concern. The strategy to choose *C. capsularis* cv. JRC 321 is that apart from being the most popular and cultivated variety in India, this variety has better characters than any other variety. Following are the characters of the *capsularis* varieties of jute.

Table 2: **Characters of the *capsularis* varieties of jute**

Corchorus capsularis L.	
JRC 321(Sonali)	Suitable for early sowing in low lying areas and fine fiber quality.
Padma	Suitable for early sowing in low lying areas.
JRC 7447	Responsive to high nitrogenous fertilizers.
JRC 212 and JRC 698	Suitable for mid-land situation.
UPC 94	Fine fiber quality
JRC 4444 and KC 1	Suitable for Orissa
KTC 1	Suitable for Bihar
Bidhan Pat 1, 2 and 3	Photo-insensitive, short duration.

2.4 Major diseases and pests

All plants are affected by various pathogens from seedling to the maturity stage. They cause considerable damage to plants, which result in crop loses. Jute, being a fiber crop, the stem is of economic value. It has been estimated that about 60% yield of jute fiber is lost due to fungal diseases every year. Major fungal pathogens causing diseases in jute are *Macrophomina phaseoli* that causes stem rot, anthracnose caused by *Colletotrichum corchori*, soft rot by *Sclerotium rolfsii*, black band by *Botryodiplodia theobromae*, die-back by *Gloeosporium* sp., powdery mildew by *Oidium* sp. Of all, *Macrophomina* is a major fungal pathogen in this crop.

Other invading pathogens include root-knot caused by a nematode (*Meloidogyne arenaria*) and leaf yellow mosaic virus (Ghosh et al., 1983).

2.5 Genetic improvement by conventional breeding in jute

Development of hybrids with improved fiber quality, better adaptability and disease resistance are the key objectives to initiate breeding programs in jute. Interspecific crosses have been initiated in jute for achieving desirable characters. A prerequisite in plant breeding is the ability of the breeding parents to produce a viable hybrid. For jute improvement, ploidy level and interspecific crosses are the major impediments. These species are highly self pollinated and have 14 diploid number of chromosomes (2n = 14), yet they are sexually isolated (Mia et al., 1967). Both of the species have their own unique characters that *capsularis* can resist flooding and *olitorius* is resistant to fungal pathogens. Thus a combination of these characteristics would develop a hybrid which has wider adaptability and resistance to fungal infections. Interspecific hybridization between the two species of *Corchorus* was first attempted by Finlow et al., (1921), but without any reported success. Since then, many attempts by researchers to cross these species failed during the past century. (Ganesan et al., 1957; Swaminathan et al., 1961; Datta et al., 1960; Datta et al., 1961; Das et al. 1972). A strong sexual incompatibility, which causes premature cessation of embryo growth, is responsible for the failure of seed set in interspecific crosses performed by Ganesan et al., (1957). Incompatibility barrier exists between these two species of jute preventing any cross breeding (Ganesan et al., 1957;

Kundu et al., 1959). Crosses made by Datta et al., (1961) between induced autotetraploids of both species in either direction with a vision to produce amphidiploids obtained tetraploid pods. But most of the set pods did not mature and shed during development. Swaminathan et al., (1961) reported to have obtained hybrids through reciprocal grafting of the two parents before crosses. But there is still a lag in commercialization of the produced hybrids as the farmers had their own doubts and the produced hybrids never reached the farmers hands (Mia et al. ,1967).

2.6 Jute tissue culture

The science of *In vitro* cell culture has taken its roots with the discovery of the technique by a german scientist Gottlieb Haberlandt in 1902. The proposals of cell theory by Schwann and Schleiden that every cell is capable and can be grown into a complete plant by supplementing proper nutrients was considered strongly by him. Thus, the concept of totipotency was proposed. The contribution of Skoog and Miller (1957) jointly demonstrated that auxin – cytokinin balance is a prerequisite in the regulation of morphogenesis *in vitro* cultures. Many other important discoveries accelerated the *in vitro* culture technique. Remarkable breakthrough in this area happened with the discovery of by Went (1926) that Indole – 3- acetic acid promotes cell growth. For the first time J. Reinhert and F.C. Steward in 1958 independently demonstrated the somatic embryogenesis in *Daucus carota* callus cultures in a reproducible manner. Toshio Murashige and Folke Skoog's (1962) efforts towards popularization of this technique is a major milestone in the science of tissue culture. A culture medium that can be used to grow any type of plant or culture was formulated by them. With the use of this culture medium and with the manipulation of auxin and cytokinins levels, *in vitro* plant regeneration became a reality.

As important as the balance between auxin and cytokinins, the plant genotype also plays a vital role in *in vitro* response of cells in tissue culture. Several reports in the past state the fact that genotypes of the same cultivar respond in an independent fashion in cell cultures towards *in vitro* regeneration. Gandonou et al.,

(2005) showed that plant regeneration capacity in sugarcane is significantly affected by genotype. Thus, screening of genotypes for *in vitro* plant regeneration is always a very important research task. Literature suggests the cultivar JRC-321(Sonali) of *C. capsularis* is one of the popular cultivars in India, has fiber fineness about 10 denier than the other released varieties that varied between 16 and 18 denier. Also, this variety can be grown with little soil moisture and can be harvested early. The present work is oriented towards using this cultivar in studies on genetic manipulation.

Micropropagation protocols for this species have been tried out in the eighties (Ahmed et al., 1989) followed by the reports on regeneration from aseptically grown seedling explants (Abbas et al., 1997; Khatun et al., 1993a, b; Saha et al., 1999; Naher et al., 2003). There was no convincing report in terms of high frequency multiple shoot regeneration and establishment of plants in the field. Sarker et al., (2007) reported regeneration in white jute in the local cultivars of Bangladesh. However, the use of the same regeneration protocol was not effective in regenerating Indian genotypes of *C. capsularis* indicating that different cultivars need optimized growth regulator combinations.

Table 3: Status of *in vitro* regeneration in *C. capsularis* L.

Sl.No	Cultivar used	Explant ; Age of explant; Medium and PGR's used	Status of regenerated plants	Reference
1	D-154	Apical meristems and plumules; Callus; BAP and tyrosine fortified medium	Obtained regeneration but suspected that it was from concealed primordial	Rahman et al., 1985
2	CVL-1, D-154	Hypocotyl segments; 7-d old; MS supplemented with tyrosine and NDGA	Studied organogenic nature of callus extensively. Produced shoots.	Seraj et al., 1992
3	D-154	Nodal segments, shoot tips, root segments, cotyledons; 10-d old; MS with 0.5 mgl^{-1} IAA and 2.0 mgl^{-1} BAP	Shoot tips and nodal segments produced maximum shoots – suggests clonal propagation	Abbas et al., 1997
4	JRC 321	Cotyledons; 2-d old; liquid culture; modified MS	Plantlets could not be established	Saha & Sen 1992

Sl.No	Cultivar used	Explant ; Age of explant; Medium and PGR's used	Status of regenerated plants	Reference
5	CVL-1, CVE-3, D-154, CC-45, Tri-cap-1, Tri-cap-2, BJC-83, BJC-718, BJC-2142, BJC-7370	Cotyledons (with attached petioles); 0.5 mgl^{-1} IAA and 2.0 mgl^{-1} BAP	Regenerated plants transferred to field. Regeneration depended on genotype. Tri-cap-1 was a high regenerative genotype.	Naher et al., 2003
6	CVL-1, CVE-3, D-154	Petiole attached cotyledons; 7-d-old; MS with 1.0 mgl^{-1} IAA and 0.2 mgl^{-1} BAP Cotyledonary nodal explants MS with 1.0 mgl^{-1} IAA and 0.2 mgl^{-1} BAP	Established plantlets in field conditions. Plants obtained from nodal explants have concealed primordial.	Sarker et al., 2007
7	CVE-3	7-d old petiole attached cotyledons; MS with 0.5 mgl^{-1} IAA and 2.0 mgl^{-1} BAP	Shoot induction was observed but no report on plantlet establishment	Begum et al., 2009

2.7 Gene transfer methods

The ability of scientists to generate fertile plants from a single cell paved the way to transgenic technology. In the early eighties, a method to surpass the limitations of selective breeding was discovered by scientists. They invented procedures for combining the DNA of species as distantly related as plants and animals and the organisms produced by such means are termed transgenic. This branch of genetic engineering made it possible to design novel organisms. The capacity to introduce and express diverse foreign genes in plants was first described for tobacco in 1984 (Horsch et al. & De Block) and has been extended to many species. The technologies used for gene transfer are vector mediated or by direct gene transfer. Both methods are briefly discussed here under.

2.7.1 Direct gene transfer/ Particle bombardment

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The technique was invented by Sanford et al., (1987) and is a rapid and simple system to deliver DNA into cells directly. Also referred to as particle gun or gen gun mediated gene transfer, this is the most widely used technique for transformation experiments after *Agrobacterium* mediated transformation method.

The principle of this technique is to deliver DNA particles into intact cells without causing any irreversible damage to the cells. During this process, pure DNA is coated over colloidal gold or tungsten particles which are microscopically small particles (1-4 μM) and accelerated to high velocities (300-600 ms^{-1}) under pressure. Vacuum is created and pressure is applied for the particles to hit the cells and penetrate directly into them. The DNA may be delivered into the chloroplasts or mitochondria or the nucleus or cytosol. Expression of the gene depends on the integration, whether stably integrated or partially. This technique is now being applied to transform other cell types like fungi, algae, *Agrobacterium* etc. This method is expensive and requires extra instrumentation. Ghosh et al., (2002) used this technique in generation of stable transgenics in jute.

2.7.2 *Agrobacterium* mediated/ vector mediated

Agrobacterium tumefaciens-mediated transformation has been widely used for research in plant molecular biology and for genetic improvement of crops since 1983 because of its simplicity and inexpensive nature. The advantage of the method is the wide host-range of the bacterium. The other merits include integration of the small copy number of T-DNA into plant chromosomes, and stable expression of transferred genes. However, even with those superior attributes, it is still difficult to achieve high reproducibility and consistency of transformation events that are prerequisites for large scale transformation experiments in plant biology. *A. tumefaciens* is capable of transferring a particular DNA segment (T-DNA) of the tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is subsequently stably integrated into the host genome and transcribed. (Binns and Thomashaw, 1988). Any foreign DNA placed between the T-DNA borders can be transferred to plant cell, no matter where it originates from. Sarker et al., (2008) transformed the mature embryos and petiole attached cotyledons by this method in the cultivars of Bangladesh. However, there was no plantlet recovery after the selection on 100 mgl^{-1} kanamycin medium. Failure to recover plantlets from selection medium supports a need to standardize the concentration of selection agent used in the medium.

Table 4: Status of transformation in *C. capsularis* L.

Sl.No	Cultivar and explant used	DNA delivery method	Selection	Transgenic plant analysis	Reference
1	JRC 321, apical meristems	Particle bombardment	*bar* gene	Stable transformed plants	Ghosh et al., 2002
2	CVL-1 and CVE-3 Mature embryo and petiole attached cotyledon	*Agrobacterium* mediated	Kanamycin	Obtained single shoots from mature embryo – confirmed by PCR amplification of *gus* and *nptII.* Shoots derived from petioles did not survive on 100 mg l^{-1} kanamycin	Sarker et al., 2008

2.8 Identification of transgenics

A key step in the plant transformation process requires the selection and recovery of the transformed shoots. Selectable marker genes have been pivotal to the development of plant transformation technologies as it allows the researchers to identify the cells that are expressing the cloned DNA and to monitor and select the transformed progeny. Since only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low (Penna et al., 2002). Since the selectable marker gene is expected to function in a range of cell types, it is usually constructed as a chimeric gene using regulatory sequences that ensure constitutive expression throughout the plant. The selectable marker gene is usually co-transformed with the gene of interest. Stringent selection of the transformed cells is an important aspect to be considered without disturbing the regeneration ability of the plant species that is transformed. The selectable marker must be able to produce notable differences between the transformed and the untransformed tissues for easy identification. Some of the selectable markers are used only to monitor the response of genes to specific stimuli. These are the scorable reporters.

2.8.1 Scorable reporter gene

The GUS reporter gene system is already a powerful tool for the assessment of gene activity in transgenic plants for quantifying the transformation efficiency. A widely used reporter gene in plants is the *uidA*, or *gus*A, gene that encodes the enzyme β-glucuronidase (GUS). This enzyme cleaves the chromogenic (color generating) substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid), resulting in the production of an insoluble blue color in those plant cells displaying GUS activity. Plant cells do not generally contain any endogenous GUS activity, so the production of a blue color when stained with X-gluc in particular cells indicates the activity of the promoter that drives the transcription of the *gus*A-chimeric gene in that particular cell. The *GUS* reporter systems have been used to improve transformation and efficiency of recovering transgenic plants by allowing the visual detection of transformed tissues. This permits the manual selection of transformed

tissues prior to the application of selection agents to transformed cells. The GUS assay is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual (Jefferson et al., 1987). Previously, *GUS* was used for in establishing transformation experiments in jute (Sarker et al., 2008).

2.8.2 Selectable marker

The most widely used selectable marker gene is the neomycin phosphotransferase gene (Fraley et al., 1983), which confers resistance to aminoglycoside type antibiotics. The neo gene from transposon Tn5 encodes a neomycin phosphotransferase II (*npt II*) and therefore confers resistance on a host cell to aminoglycoside antibiotics including kanamycin, gentamycin and neomycin. Kanamycin is very effective in inhibiting the growth of untransformed cells.

The *bar* gene from *Streptomyces hygroscopicus* encodes phosphinothricin acetyl transferase (*PAT*), that provides resistance to herbicide basta (Thompson et al., 1987). Phosphinothricin inhibits glutamine synthase activity that leads to cell death due to accumulation of ammonia. *bar* gene has been used in many of the crops successfully. Bialaphos is now being used in agriculture as a non-selective herbicide. It is a tripeptide which is composed of two L-alanine residues and an analogue of glutamic acid known as phosphinothricin (PPT). PPT is an inhibitor of glutamine synthetase in plants and bacteria, the intact tripeptide has little or no inhibitory activity *in vitro (*De block et al., 1987). In both bacteria and plants, intracellular peptidases remove alanine residues and release active PPT. *S. hygroscopicus* is used for the commercial production of bialaphos.

2.9 Progress in jute protoplast studies

Due to increasing interest in the diversified products of jute, there is a continuous demand for new cultivars with elite traits with respect to fiber quality. For any crop improvement strategies, conventional breeding is the most suitable in many crops. But, in jute due to the presence of a strong genetic barrier between the two species, for enhancing genetic variability through introgression, it necessitates the use of an alternative strategy. Breeders are still looking for new techniques to transfer genes of interest to jute cultivars. One such technique, used successfully in other crops to overcome sexual incompatibility, is protoplast fusion (Assani et al., 2005). Protoplast fusion requires the development of a regeneration system from protoplasts. Furthermore, an efficient and reliable regeneration system from protoplasts opens up new opportunities for genetic transformation via direct DNA uptake or electroporation, as was shown in many other crops (Potrykus et al., 1990). Though there are a couple of reports on somatic hybridization in jute by Saha et al., (2001), the technique was used for cell line studies and no plantlet development was obtained from the protoplast derived calli. The report on somatic embryogenesis by Saha and Sen (1992) produced embryo like structures that never developed into plantlets. The importance of protoplast research in this plant is realized late. A focused approach towards isolating and culturing of protoplasts needs attention for crop improvement in this species aiming at interspecific hybridization.

Chapter 3

CHAPTER 3

3.1 INTRODUCTION

Corchorus capsularis, white jute is widely cultivated over the Gangetic plains of North-eastern India. It is a fiber (bast) yielding cash crop with versatile uses in industry. From conventional sacking to modern lifestyle products, jute products have been used diversely in various industries with broad uses. Jute yarns are used for the manufacture of (i) high quality blended apparel grade textiles, (ii) Technical, industrial and home textiles including non-woven, (iii) automotives, (iv) soil savers, (v) bio-composites, (vi) pulp and paper, (vii) fine chemicals, cosmetics and healthcare products, and (viii) bio-fuels.

It is the second cheapest fiber after cotton in production. Aiming at mass propagation and further quality improvement, micropropagation protocols for this species have been tried out in the eighties (Ahmed et al., 1989) followed by the reports on regeneration from aseptically grown seedling explants (Abbas et al., 1997; Khatun et al., 1993a; Khatun et al., 1993b; Saha et al., 1999; Naher et al., 2003). However, there was no convincing report in terms of high frequency multiple shoot regeneration and establishment of plants in the field. It was reported earlier that *in vitro* regeneration in this genus is genotype-dependent and the material is recalcitrant to regeneration (Naher et al., 2003, Sarker et al., 2007). There are reports of regeneration in Bangladeshi varieties of tossa jute (*C. olitorius*) (Khatun et al., 2003; Huda et al., 2007; Khatun et al., 1992). Sarkar et al., (2007) reported regeneration in white jute in the local cultivars of Bangladesh. However, the use of the same regeneration protocol was not effective in regenerating Indian genotypes of *C. capsularis* indicating that different cultivars need varied growth regulator combinations. Despite the reports on regeneration from the cotyledonary petioles of *C. capsularis* of the varieties of Bangladesh, there still remains genotype dependence and regeneration in Indian variety of jute (*C. capsularis*) still remains a challenging task. However, protocols for high frequency plant regeneration have not been reported in the white jute cultivar JRC 321. Hence, the present study is undertaken

to develop an efficient and reproducible fertile plant regeneration system from cultured tissues at high frequency.

3.2 MATERIALS AND METHODS

3.2.1 Seed surface sterilization and seed germination

The seeds (*C.capsularis* L.) used in this study were obtained from CRIJAF (Central Research Institute of Jute and allied Fibers, Barrackpore, Kolkatta, India). Mature seeds were surface sterilized with 70% EtOH for I min followed by 0.1% (w/v) mercuric chloride for 3 min and rinsed five to six times with sterile double distilled water. The surface sterilized seeds were inoculated on seed germination medium (MS + 1% sucrose) and incubated in dark for 2 days and later transferred to light.

3.2.2 Identification of a responsive explant from various cultivars of jute (*C. capsularis* L.)

Different explants like cotyledons, hypocotyls, root segments, cotyledonary petiole isolated from the popular jute (*C.capsularis* L.) cultivars were tried for their response towards regeneration. JRC 212, JRC 321, JRC 7447 and JRC 698 were considered to study their response towards regeneration and the best responsive explant and cultivar were chosen for further studies.

3.2.3 Chemicals

Major and minor inorganic compounds used in basal media were purchased from Himedia (Mumbai, India). Analytical grade chemicals were obtained from Qualigens (Mumbai, India). Hormones were purchased from Sigma (USA).

3.2.4 Stock Solutions

Hormone stocks: The following auxins and cytokinins are used in the present study.

NAA: 50 mg of NAA (α-naphthalene acetic acid) was weighed and dissolved in a small volume of 1N NaOH and made up to a final volume of 50 ml with sterile distilled water and stored at 4 ^{o}C.

IAA: 50 mg of IAA (α-indole acetic acid) was weighed and dissolved in a small volume of 1N NaOH and made up to a final volume of 50 ml with sterile distilled water and stored at 4 ^{o}C.

2,4-D: 50 mg of 2,4-D (2,4 – dichlorophenoxyacetic acid) was weighed and dissolved in a small volume of 1N NaOH and made up to a final volume of 50 ml with sterile distilled water and stored at 4 ^{o}C.

Picloram: 10 mg of picloram 2,4-D (2,4 – dichlorophenoxyacetic acid) was weighed and dissolved in a small volume of 1N NaOH and made up to a final volume of 10 ml with sterile distilled water and stored at 4 ^{o}C.

BAP: 50 mg of BAP (6-Benzylaminopurine) was weighed and dissolved in a small volume of 1N NaOH and made up to a final volume of 50 ml with sterile distilled water and stored at 4 ^{o}C.

Kinetin**:** 10 mg of BAP (6-Benzylaminopurine) was weighed and dissolved in a small volume of 1N NaOH and made up to a final volume of 10 ml with sterile distilled water and stored at 4 ^{o}C.

3.2.5 Culture medium

Murashige and Skoog (1962) medium is used for seed germination and for plant regeneration. pH was adjusted either with 1 N HCl or 1 N NaOH to 5.75 – 5.8. Double distilled water was used in all media preparations.

3.2.6 Preparation of explants from aseptically grown seedlings

Hypocotyls: The apical region of the plumule is eliminated and 1 cm length hypocotyls were cultured on different hormonal combinations.

Cotyledons: Cotyledons are isolated from sterile seedlings by holding the hypocotyl with a forceps and cutting the cotyledons eliminating the apical meristems.

Mature embryos: Sterilized seeds are allowed to imbibe water for 24 hrs. The seed coat is carefully removed. A slight incision is made with a scalpel and the embryo is isolated carefully without damaging it by holding the cotyledon properly.

Cotyledonary petiole: Five day old seedlings were used for the experiments. While preparing the cotyledonary petiole explants, the pre-existing axillary bud was carefully excluded by cutting the petioles at least 1 mm away from the axil. Further, all those explants that developed a single healthy shoot within five days of culture

were eliminated to avoid the proliferation of the explants with the pre-existing meristem. The explants thus obtained were cultured on media with different growth regulator combinations by inserting the petiole regions in the medium. A minimum of 50 explants were cultured in each experimental set that was replicated three times.

3.2.7 In vitro plant regeneration from seedling derived cotyledonary petiole explants

3.2.7.1 In vitro regeneration experiments

For the initial experiments, various combinations of growth regulators were used on MS (Murashige and Skoog, 1962) medium along with 3% (w/v) or 3.6% (w/v) sucrose. The pH was adjusted to 5.8 and the medium was solidified with 0.8 % agar (Himedia, India). For initial experiments, BAP at concentrations of 0.5 – 4 mg l^{-1} was used along with auxins like NAA, IAA and 2, 4 - D at concentrations of 0.1 and 0.5 mg l^{-1} (**Table 5**). All the cultures were incubated at 27± 1 ^{o}C in the culture room with 16/8 h photoperiod under white fluorescent light. The cultures were sub-cultured every 15 days onto fresh medium to avoid browning of the tissues. The petiole explants were cultured on the most promising medium by inserting the cut end of petiole into the medium.

3.2.7.2 Establishment of regenerated plants

Shoots obtained were separated from the shoot clump. BAP in combination with GA_3 and GA_3 alone were used for shoot elongation. Once the shoots elongated to two cm, they were transferred to rooting medium. IBA, NAA and IAA at concentrations of 0.1, 0.5 and 1.0 mg l^{-1} were tested for rooting along with nutrient medium without any growth regulators. Rooted shoots were washed thoroughly with sterile double distilled water to remove any traces of medium over the roots and transferred to a mixture of soil and vermiculite (1:3) in tea cups and covered with polythene bags to maintain humidity. They were watered as and when required. They were maintained at the same culture conditions for 10 – 15 days till they developed new

leaves and later transferred to pots containing sand soil mixture (1:1) with added organic manure and were grown to maturity in the green house.

3.2.8 Histological examination

To observe the origin of shoot buds from cotyledonary petiole, the explants during the culture were periodically fixed in formalin, acetic acid, 70% alcohol (FAA 1: 1: 18) for 48 h, dehydrated through a graded alcohol series of ethyl alcohol and tertiary butyl alcohol and , embedded in paraffin (55-60^{o}C). Serial sections of 8-10µ thickness were cut with a rotary microtome and the tissue sections were stained with Hematoxylin and Eosin following standard procedure for microscopic observations.

3.2.9 Optimization of the age of seedlings

Age of the seedlings at the time of explanting was studied to analyze its effect on shoot regeneration. Explants from 5 to 11-day- old seedlings were taken from aseptically grown seedlings for their regeneration ability.

3.2.10 Data collection and statistical analyses

The regeneration efficiency was calculated as the percentage number of explants giving rise to shoots in relation to the total explants cultured. All the experiments were replicated four times with 50 explants in each replication. All the data were analyzed for analysis of variance (ANOVA) using SPSS package and the treatment means were compared using sigma plot9.0 software. All experiments were carried in a completely randomized design.

3.3 RESULTS

3.3.1 Comparison of response of different genotypes in tissue culture

Different genotypes need different growth regulator combinations for different explants (Naher et al., 2003; Sarkar et al., 2007). Hence, it is very important to identify a potential explant from a cultivar that is highly responsive to tissue culture manipulations. The most popular cultivars of the *capsularis* variety – JRC 321, JRC 212, JRC 698 & JRC 7447 showed varied responses in cultures.

Different combinations of growth regulators in varying concentrations with auxins [NAA (0.1 & 0.5 mg l^{-1}), IAA (0.1 & 0.5 mg l^{-1}), 2,4-D (2.0 – 10 mg l^{-1}), picloram(0.05, 0.1 & 0.5 mg l^{-1})] and cytokinins [BAP (0.5, 1.0 & 2.0 mg l^{-1}), kinetin (0.5, 1.0 & 2.0 mg l^{-1}), TDZ(0.01 & 0.05 mg l^{-1})] when tried to check their response on different explants like root segments, cotyledons, hypocotyls and cotyledonary petioles produced varying results. All of the 98 combinations were tried on all the explants for their response in cultures.

Root segments in culture medium turned brown in a few days of culture. Very little callus that turned quickly brown developed over the root segments in culture. Cotyledon explants developed white to yellow callus in most of the media supplemented with auxins like NAA, IAA, 2,4-D and picloram along with BAP, kinetin and TDZ as cytokinins. This callus when sub-cultured developed more callus but never differentiated into organogenic pathway. Hypocotyl explants developed callus which was fragile. Necrosis was observed upon subculture of this callus. Rarely, hypocotyls of JRC 321 produced pale green callus that gave sporadic shoot like structures which never developed into true shoots. In contrast, hypocotyls of JRC 698 cultivar produced white to green callus which later gave rise to multiple shoot regeneration. Cotyledonary petioles of JRC 321 developed shoot buds on 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BAP supplemented medium. Cotyledonary petioles of JRC 321 proved to be the best source of explants for further manipulations with the highest response of 67.8% regeneration efficiency. Hence all the experiments in the present study focused on using the genotypes JRC 321 or JRC 698.

The response of cotyledonary petioles of JRC 321 in cultures and hypocotyls of JRC 698 were discussed elaborately in separate sections in this study.

3.3.2 Effect of plant growth regulators on different explants of cultivar JRC 321

3.3.2.1 Hypocotyls as source of explants

The response of hypocotyl segments on various growth regulator combinations was studied. The basal medium without plant growth regulators was used as a control. Callus developed on the hypocotyl segments in 2-3 weeks, while there was no callus formation on the control explants. Hypocotyl explants remained green on BAP containing medium when compared to TDZ. On a medium containing TDZ alone, white hard callus developed which could not develop further. Since, on a BAP containing medium there was callus development which was green and granular, all further experiments were carried using BAP.

2, 4 – D did not have any effect on shoot bud induction with various combinations of cytokinins like BAP, Kinetin and TDZ. IAA induced less compact and pale green callus which turned brown and showed only limited growth. Callus produced with IAA in combination with BAP was pale, yellowish green in color, which later turned brown and incapable of differentiation (**Fig 3a & 3b**).

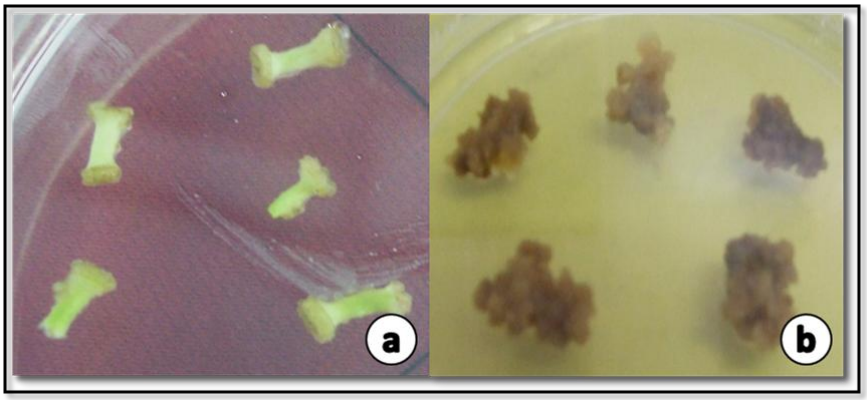


Fig 3: Response of hypocotyl cultures on BAP and IAA containing medium: (a) Callus developing on hypocotyls on IAA and BAP medium. (b) Callus turned brown upon continued culture on IAA and BAP medium.

3.3.2.2 Cotyledons as source of explants

The first two leaves developed from germinating seeds were used in our experiments. Cotyledons cultured on different growth regulators yielded callus. Sporadic shoots were observed on NAA and BAP containing medium **(Table 5)**. The callus was white and fragile on IAA medium which did not proliferate further. Picloram produced yellow to white callus that sometimes developed leaf like structures rarely and soon necrotized. Two to five day old cotyledons were cultured on NAA and BAP medium. The two day old cotyledons gave a better response than the older explants by producing little callus on the lamina. We could observe globular white to green calli towards the edges of the lamina on most of the cultured cotyledons (**Fig 4a**). This callus, when cultured separately on medium containing various combinations of hormones did not yield any further proliferation. Few of the explants when sub-cultured on different media without separating the small bud like calli, soon necrotized and turned brown (**Fig 4b**). Though the technique of liquid culture was used to regenerate cotyledon explants by Saha and Sen (1999), we could not reproduce the same.

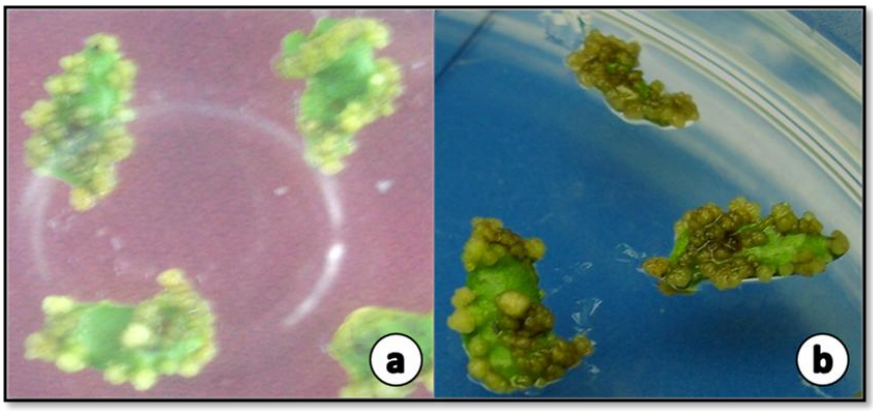


Fig 4: Response of cotyledon explants on NAA and BAP containing medium: (a) Callus developing on lamina. (b) Callus turned brown in further sub-cultures.

Table 5: Effect of growth regulators on cotyledon explants of jute (*C.capsularis* cv. JRC 321)

AUXINS		CYTOKININS							
		BAP			Kinetin			TDZ	
		0.5	1.0	2.0	0.5	1.0	2.0	0.01	0.05
IAA	0.1	nil	nil	nil	wc	wc	wc	wc	wc
	0.5	nil	nil	nil	wc	wc	wc	wc	wc
NAA	0.1	Expanded	Callus	Callus	nil	nil	nil	wc	wc
	0.5	Expanded cotyledons	Callus towards edges	Sporadic shoots	nil	nil	nil	wc	wc
2,4-D	2.0	nil	nil	nil	yc	yc	yc	wc	wc
	4.0	nil	nil	nil	yc	yc	yc	wc	wc
	5.0	nil	nil	nil	yc	yc	yc	wc	wc
	10.0	nil	nil	nil	yc	yc	yc	wc	wc
	20.0	nil	nil	nil	yc	yc	yc	wc	wc
Picloram	0.05	yc	yc	yc	nil	nil	nil	nil	nil
	0.1	yc	yc	yc	nil	nil	nil	nil	nil
	0.5	yc	yc	yc	nil	nil	nil	nil	nil

wc – white callus; yc – yellow callus

3.3.2.3 Mature embryo as source of explants

Embryos behaved differently from those of cotyledons and hypocotyls. Upon culture of the embryos on TDZ, they first formed the primary shoot axis and subsequently from the surface of the embryo developed the multiple shoots (**Fig 5a**). The multiple shoots developed from the surface of the embryo could be clearly distinguished from the primary shoot. This nature of shoot formation was a rare event and the formed shoots were also not healthy. These shoots appeared fasciated and clumped. Shoots could not be elongated as they fell separate when separating from the shoot clump..

However, sometimes dark green globular structures emerged from the callus. Upon culture of the mature embryos on 0.05 mgl^{-1} TDZ in dark for three months, white globular callus could be observed. Sequential sub-culture of this callus on 0.05 mgl^{-1} BAP and 0.1 mgl^{-1} NAA along with 0.5 mgl^{-1} BAP in light, yielded green globular structures resembling somatic embryo like structures(**Fig 5b, 5c**). These embryoids developed leaf like structures over them (**Fig 5d**). These leaf-like structures were flat. Elongating these structures was futile as they soon turned brown upon subculture. The other globular structures remained green on NAA- BAP medium and no further progress is observed in their development.

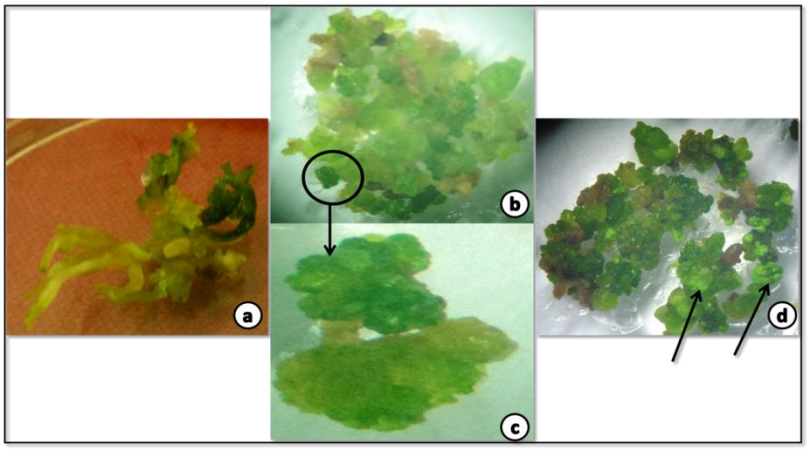


Fig 5: Embryos cultured on TDZ containing medium (a) Multiple shoots developing from the embryo. (b) Green globular callus developed from mature embryo. (c) Enlarged view of the globular nature of the callus. (d) Callus developing leaf like structures.

3.3.3 Response of cotyledonary petiole explants towards treatment with plant growth regulators for *in vitro* plant regeneration

3.3.3.1 Shoot regeneration

Multiple shoot regeneration was observed at the distal cut ends of the cotyledonary petiole explants after three weeks of culture on the regeneration medium. Shoot buds appeared after two weeks and shoots were visible by 21-days of culture on the medium. A combination of 0.5 mgl^{-1}NAA and 0.5 mgl^{-1} BAP with 36 gl^{-1} sucrose (termed as regeneration medium) promoted 67.8% shoot regeneration frequency with shoot bud differentiation at the distal cut end of the cotyledonary petiole. Shoot buds developed directly from the junction of the petiole and cotyledonary lamina **(Fig 6a-f, Fig 7f)**. They also developed from the callus produced at the cut ends and were glossy, dark green in nature. No shoot regeneration was noticed at lower concentration of NAA with various BAP levels **(Table 6)**. An average of 5-6 shoot buds developed initially. Up on sequential subculture to 0.3 mgl^{-1} and 0.1 mgl^{-1} BAP containing medium, the number of shoot buds could be increased to an average of 10 per explant.

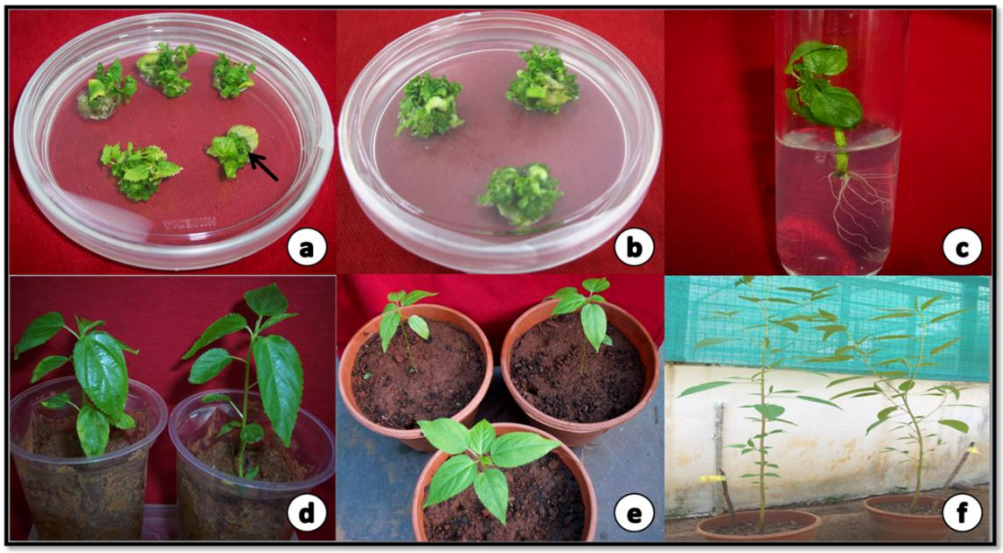


Fig 6: Plant regeneration by organogenesis from cotyledonary petiole explants in Jute (*Corchorus capsularis* L.*)* cv. JRC 321 (a) Origin of shoot buds at the junction of cotyledon and petiole (indicated by an arrow) with intact cotyledon. **(b)** Proliferation of shoots. **(c)** Rooting of regenerated shoots. **(d)** Acclimatized and hardened plantlets. **(e)** Well rooted plants transferred to green house. **(f)** Mature plants bearing flowers and pods.

In media supplemented with NAA (0.1, 0.5 mg/ l) and BAP (0.5, 1.0, 2.0, 4.0 mg/ l), white to green fragile callus developed from the explants. Subsequent subculture of this callus did not lead to any shoot bud development. NAA was more suitable for callus formation. However, no difference was observed in callus induction by the addition of 2,4 - D regardless of the concentration of BAP used (0.5, 1.0, 2.0, 4.0 mgl^{-1}) **(Table 6)**. Calli obtained on media containing IAA and BAP were soft and light brown. 2,4-D was also not able to induce shoot regeneration in combination with BAP on the explants of this cultivar. The hypocotyl-derived calli on NAA and BAP were green in color with the formation of green nodular structures after six weeks of culture. However, these nodular structures did not proliferate further into embryo or shoot-bud like structures. These observations indicate that NAA influences callus induction in tissue cultures of *C. capsularis* (JRC 321) and that a combination of NAA and BAP is promising for shoot bud induction in petiole explants.

Table 6: Effect of IAA, NAA, 2,4 D and BAP on hypocotyls and cotyledonary petiole explants of Jute (*C. capsularis* cv. JRC 321) in shoot bud induction after four weeks of incubation [*1]

Growth Hormones			**BAP (mgl^{-1})**			
Auxin (mgl^{-1})			0.5	1.0	2.0	4.0
NAA	0.1	H	0[*2]	0	0	0
		P	11.66±0.66[*3]	9±0.57	8.33±0.33	0
	0.5	H	0	0	0	0
		P	20.33±0.33	16.33±0.33	9.66±0.33	8.66±0.33
IAA	0.1	H	0	0	0	0
		P	0	0	0	0
	0.5	H	0	0	0	0
		P	0	0	0	0
2,4-D	0.1	H	1[*4]	1	1	1
		P	1	1	1	1
	0.5	H	1	1	1	1
		P	1	1	1	1

H- Hypocotyl; P- Cotyledonary petiole

[*1] 30 explants in triplicates ; [*2] Indicate profuse callusing; [*3] Data indicates Mean ± SE; [*4] No callusing and no response

3.3.3.2 Histological analysis

Cross sections of the explants that were fixed at different stages revealed that the regenerated buds developed from the epidermal and sub-epidermal cells and these cells divided initially followed by the divisions in the inner rows of cells and the stimulated cells beneath developed shoots. The gradual development of shoot buds is shown in **Fig 7 (a-f)**. The developed shoot notch could be clearly observed after 9th day of inoculation. Well formed shoots could be observed by 15th day of the culture.

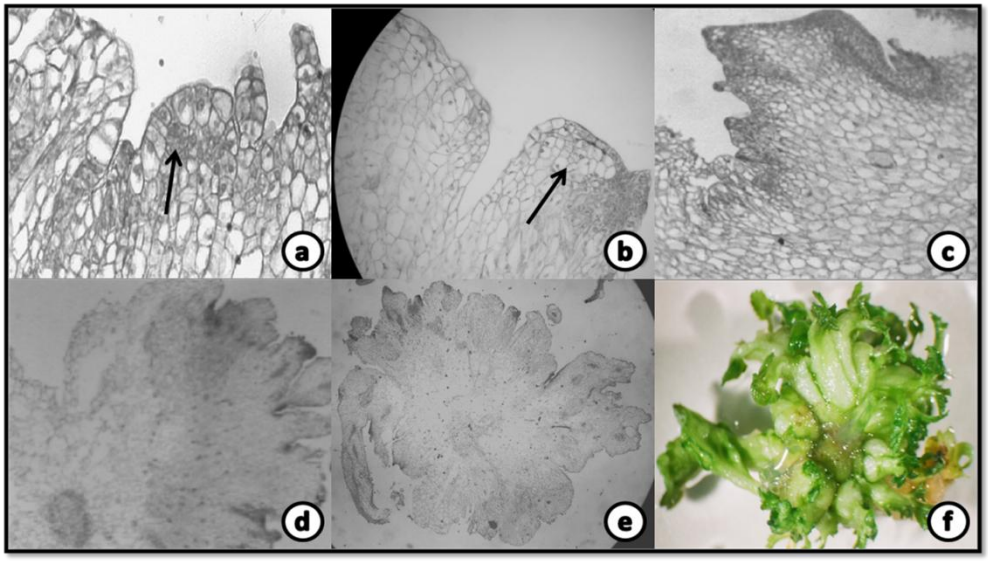


Fig 7: Histological analysis of emergence of shoot buds at cotyledonary petiole **(a)** First divisions of epidermal cells (40×) **(b)** Further divisions to the inner row leading to a bud-like projection (40×) **(c)** Well formed shoot bud projection (10×) **(d)** Shoots formed which are connected to the cambium (10×) **(e)** Horizontal sectional view of the explant which has multiple buds (10×) **(f)** Regenerated shoots from the abaxial side of the explant as seen under stereo-zoom microscope.

3.3.3.3 Effect of vitamins on multiple regeneration of cotyledonary petioles:

MS vs B_5

We noticed that the averages for both vitamins differed significantly (**Table 6**). Also the standard deviation differs in a similar manner. To establish this fact, we have computed the Students two sample t-test and found that there was significant difference between explants cultured on media containing MS and B5 vitamins. From the values of standard error of means, it could be observed that the response of regeneration obtained on MS vitamins was better than the composition of B_5 vitamins (**Table 7**).

Table 7: Influence of vitamins on shoot regeneration

Target	N	Mean	Std. Deviation	Standard error
MS vitamin	4	33.5000	0.5774	0.2887
B_5 vitamin	4	23.2500	0.9574	0.4787

3.3.3.4 Effect of age of seedling

The age of the donor seedlings and regeneration capacity showed an inverse relationship with the percentage of cultures showing shoot regeneration. In all the experiments aimed at testing the effect of the age of explants, the number of explants producing shoot buds declined with the increase in age of the seedlings acting as donors for the petiole explants **(Fig 8)**. As far as the seedling age is concerned, explants from 5-day old seedlings showed the highest response. Also, we did not notice significant variation between 5 and 7-day old seedlings. Thereafter, there was a significant decline in shoot bud differentiation with increased age of seedlings acting as explant donors.

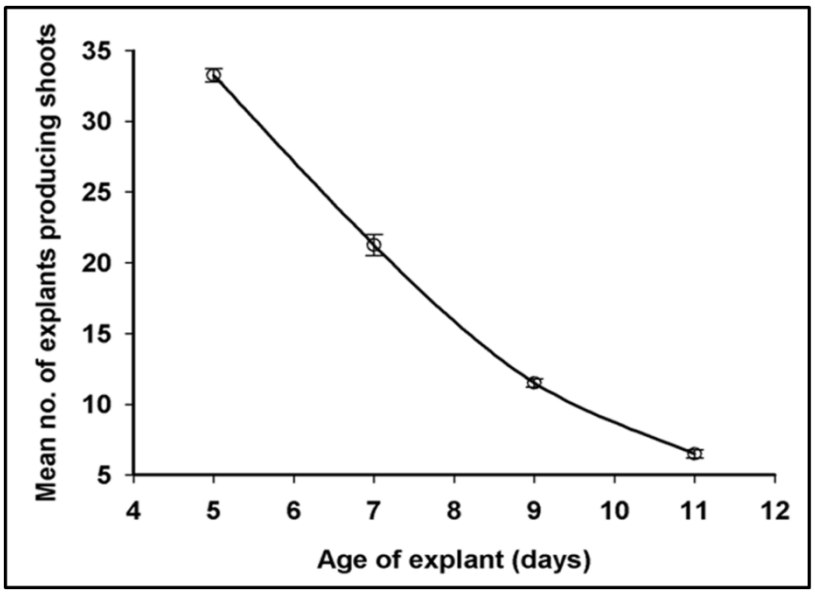


Fig 8: Graph illustrating the effect of age of seedling with the mean number of explants producing shoots

The *F*-statistics of shoot bud induction showed that the age of the seedling had a significant effect on shoot bud induction (p= 0.000). The day-wise performance evaluated by multiple-comparison test indicated that the difference in the mean regeneration frequencies between 5 and 7-day old explants was not significant **(Table 8)**. However, the number of shoot buds produced on explants from 5-day old seedlings was significantly higher compared to the explants prepared from 7-day old seedlings. These observations clearly suggest that the 5-day old seedlings are the best source for generating the cotyledonary petiole explants in *C. capsularis.*

Table 8: Multiple comparison (*F*-statistics of shoot bud induction)

(I) days	(J) days	Mean difference (I-J)	Std. Error	Sig.
5.00	7.00	12.0000*	0.6922	0.000
	9.00	21.7500*	0.6922	0.000
	11.00	26.7500*	0.6922	0.000
7.00	5.00	-12.0000*	0.6922	0.000
	9.00	9.7500*	0.6922	0.000
	11.00	14.7500*	0.6922	0.000
9.00	5.00	-21.7500*	0.6922	0.000
	7.00	-9.7500*	0.6922	0.000
	11.00	5.0000*	0.6922	0.000
11.00	5.00	-26.7500*	0.6922	0.000
	7.00	-14.7500*	0.6922	0.000
	9.00	-5.0000*	0.6922	0.000

***The mean difference is significant at 0.05 level

All the data were analyzed using analysis of variance (ANOVA) and the treatment means were compared using SPSS package and were done in a completely randomized design.

3.3.3.5 Shoot elongation and rhizogenesis

Multiple shoots developed on media supplemented with 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} BAP. BAP (0.2 mgl^{-1}) in combination with 0.5 mgl^{-1} GA_3 was used for rapid shoot elongation. The elongated shoots were of 1-2 cm in length, when they were subcultured for root initiation. When GA_3 alone was added at 1.0 mgl^{-1}, necrosis was observed. Shoot elongation was not synchronous in these explants. The elongated shoots were separated from shoot clumps and transferred to root induction medium without any growth regulators. Rhizogenesis was observed from the regenerated shoots two weeks after transfer of shoots to MS medium **(Fig 6c)**. Acclimatized plants were transferred to pots containing soil and were grown to maturity in the green house **(Fig 6 d-e)**. On transfer to soil, the regenerated plantlets survived and appeared to be morphologically similar to the normal seed-grown plants. They developed pods and set fertile seeds **(Fig 6f)**.

3.4 DISCUSSION

In the present study, we succeeded in obtaining short term protocol for high frequency plant regeneration from cotyledonary petiole explants as done previously in radish (Murakami et al., 1995), Indian mustard (Singh et al., 2009). Development of simple method to regenerate fertile plants in short periods of time is a pre-requisite for genetic modification in important cash crops like jute. Plant regeneration using hypocotyl segments (Ghosh and Chatterjee, 1990) as explants was reported in *C. olitorius*. But Huda et al., (2007) found this system not efficient for further exploitation. In our studies, using different combinations of NAA-BAP; IAA-BAP and 2,4-D- BAP, there was either callus development or no response of the hypocotyl segments. Callus that developed on the explants turned brown in further subculture. It was shown that petiole attached cotyledon was most suitable for inducing multiple shoots in the local cultivars of white jute in Bangladesh (Sarker et al., 2007). In the present study, different plant growth regulators were used in regeneration experiments. Cotyledonary petioles upon culture on medium supplemented with IAA (0.1 & 0.5 mgl^{-1}) in combination with BAP (0.5 – 4 mgl^{-1}) developed callus initially, but later necrosis of callus was visible. We found that either hypocotyls or cotyledonary petiole explants cultured on 2,4 –D alone or in combination with BAP (0.5 – 4 mgl^{-1}) had no signs of callus development. The cotyledons attached to the petioles turned brown and fell off the petioles.

Cotyledons have been a good source of multiple regeneration in many plants like peach (Mante et al., 1989), sunflower (Ceriani et al., 1992), bottle gourd (Han et al., 2004). Saha and Sen (1999) reported multiple shoot induction from jute cotyledons cultured in liquid media. There was a peculiar response of cotyledons in culture that they produced good green globular callus towards the outer edges of the cotyledons when cultured on NAA-BAP medium. However, this callus did not survive upon subculture. Mature embryos also produced dark green globular callus on TDZ medium. This callus upon subculture produced leaf like structures which soon underwent necrosis in subsequent passages.

Cotyledonary petioles as explants gave shoot bud induction in NAA- BAP containing medium. The stimulatory effect of NAA-BAP has been earlier reported to facilitate regeneration in some of the cultivars of jute. Sarker et al., (2007) obtained multiple shoot regeneration in the local cultivars of Bangladesh using combination of 1.0 mgl^{-1} IAA and 0.2 mgl^{-1} BAP for the cultivar CVL-1 and D-154. On the other hand, CVE-3 showed best response on 0.5 mgl^{-1}NAA and 2.5 mgl^{-1} BAP. Their investigation showed the differential responses among the varieties and also stated the fact that different genotypes need different combinations and concentrations of growth regulators. In the present study, we achieved 67.8 % regeneration efficiency using cotyledonary petiole explants with 0.5 mgl^{-1} NAA and 0.5 mgl^{-1} BAP as PGR's. Histological studies provided evidence for *de novo* organogenesis from the petiole explants.

We observed that age of the explant played a major role in organogenesis. Khatun et al., (2003) used 7 day old seedlings for obtaining organogenesis from petiole explants in kenaf. Begum et al., (2009) used 7 day old seedlings for regenerating *C. olitorius* petiole explants. We observed that age has an inverse relationship with regeneration efficiency. Hence, we chose to use 5 day old seedlings in comparison to 7 day old seedlings to isolate cotyledonary petiole explants.

In conclusion, the findings evidence an efficient protocol for *in vitro* plant regeneration from cotyledonary petiole explants of a popular white jute cultivar JRC 321. The present protocol provides a rapid regeneration system that could be reproduced and can be used for genetic modification in this crop species.

3.5 CONCLUSION

Though there are several reports on shoot bud regeneration from the cotyledonary petioles of some genotypes in *C. capsularis*, none of them dealt with the production of complete plantlets except the report of Sarkar et al., (2007) in the local cultivars of Bangladesh. The protocol discussed could not be applied to Indian jute cultivars. To the best of our knowledge, this is the first report of an efficient regeneration system using cotyledonary petioles of a local Indian variety with high frequency of regeneration. The present reported protocol is simple and rapid, and leads to the establishment of *in vitro* regenerated plantlets efficiently under greenhouse conditions. We have also shown that the shoot buds were regenerated *de novo* on the explants after culture initiation through histological analysis.

Chapter 4

CHAPTER 4

4.1 INTRODUCTION

Jute has been under cultivation in India for nearly two centuries now (Banerjee et al., 1955). Raw jute has been traditionally in use as a source of raw material for packaging industry only so far. In the recent times, its importance as a versatile source for diversified application, in the textiles industry, in the paper industry, in building and automotive industry, as soil saver, as decorative & furnishing material, etc. have been recognized and its demand in a number of countries is on the rise at an increasing rate. As such demand for natural fibers is on the increase for environmental concerns, therefore increase in productivity of jute crop assumes much greater importance in the coming decades. In view of the growing demand for quality fibers needed for manufacturing more diversified products, development of varieties having better fiber quality to meet specific end use requirement is to be augmented. An important cash crop of the Indian subcontinent, crop improvement of jute remains a challenge due to the recalcitrance of the plant towards genetic modifications.

An efficient transformation system would rely on the generation of stable fertile transgenic plants. Till date, there are no advancements in the genetic modification of this crop as the plant shows high recalcitrance to tissue culture and *Agrobacterium* infection. There are few trails of genetic transformation on the tossa and deshi varieties in Bangladesh. The reports on the genetic transformation of jute are scanty and inconsistent. However, there are limited numbers of reports on the recovery of *Agrobacterium*-mediated transformed (transgenic) jute plants (Sarker et al., 2008). Major drawback to use these protocols was either the protocols are purely genotype dependent or there were no reports of fertile transgenics or the protocols were not reproducible. The type of explant is also an important factor and it must be suitable for regeneration allowing the recovery of whole transgenic plants (Gustavo et al., 1998). The published protocols for transforming the *capsularis* varieties in Bangladesh used petiole attached cotyledons or mature embryos in the

transformation experiments (Sarker et al., 2008). However, the plantlets produced were unable to survive on selection regime followed and no plants were obtained on the selection medium. The only viable method developed so far in the production of stable fertile transgenics used the method of particle bombardment (Ghosh et al., 2002) of apical meristems. Considering the constraints in the method, *Agrobacterium* mediated transformation appears to be a better option, which is a simple natural gene transfer system. The method has advantage that it can stably transfer large pieces of DNA with minimal rearrangements of the transferred DNA. Also, it is cost effective, commonly used and has high efficiency of transformation. The optimization of *Agrobacterium tumefaciens*-plant interaction is probably the most important aspect to be considered. The *Agrobacterium*-mediated transformation protocols differ from one plant species to other and, within species, from one cultivar to other (Gustavo et al., 1998).

Agrobacterium strain, co-culture and inoculation conditions, *vir* gene induction, plant genotype, application of selection agents are some of the factors that need to be considered in the present study. The transfer of *β-glucoronidase* gene in the co-cultivated explants and monitoring its expression was used for optimizing the parameters for efficient transformation procedure that could be further used in transformation experiments.

For the identification of lines that have the transgene, a selection regime is to be followed. Marker genes identify the cells that express the cloned DNA and select the transformed progeny. Selection regimes using different selective agents need to be optimized for their further application.

The present study was undertaken to transform tissues with *Agrobacterium* and to identify the transgene integration. The difficulties in this process are discussed and alternate methods are discussed in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Marker genes for plant transformation

Plant transformation technologies rely on the use of selectable markers genes that are co-introduced with the gene of interest to identify or select the transformation events. Since only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Valuable companion to the selectable marker genes are the reporter genes, which do not provide a cell with a selective advantage, but which can be used to monitor the process of transformation and manually separate transgenic tissues from the non-transformed ones.

4.2.1.1 Scorable reporter genes

A widely used reporter gene in plants is the *uidA*, or *gus*A, gene that encodes the enzyme β-glucuronidase (GUS). This is used for analyzing the treated explants or plants for transformed sectors and follow the process of transformation. This enzyme can cleave the chromogenic (color generating) substrate X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid **(Fig. 9)**, resulting in the production of an insoluble blue color in those plant cells displaying GUS activity. Generally, plant cells do not contain any GUS activity except some legumes, so the production of blue color when stained with X-gluc in particular cells indicates the activity of the promoter that drives the transcription of the *gus*A-chimeric gene in that particular cell. The GUS assay is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual (Jefferson et al.,1987).

In all the experiments with cotyledonary petiole explants as well as mature embryos subjected to GUS assay, the tissues were incubated in the reaction buffer containing 50 mM NaH_2PO_4 (pH 7), 0.01% Tween 20, 10 mM Na_2EDTA and 0.3% (w/v) 5-bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. Tissue is incubated at 37^{o}C overnight and screened for blue staining indicating *gus* activity under a stereo-microscope. A short treatment with methanol is carried out before

visual examination to intensify the blue staining as methanol removes the chlorophyll in the cells which interferes with the observations on the blue colored gus spots.

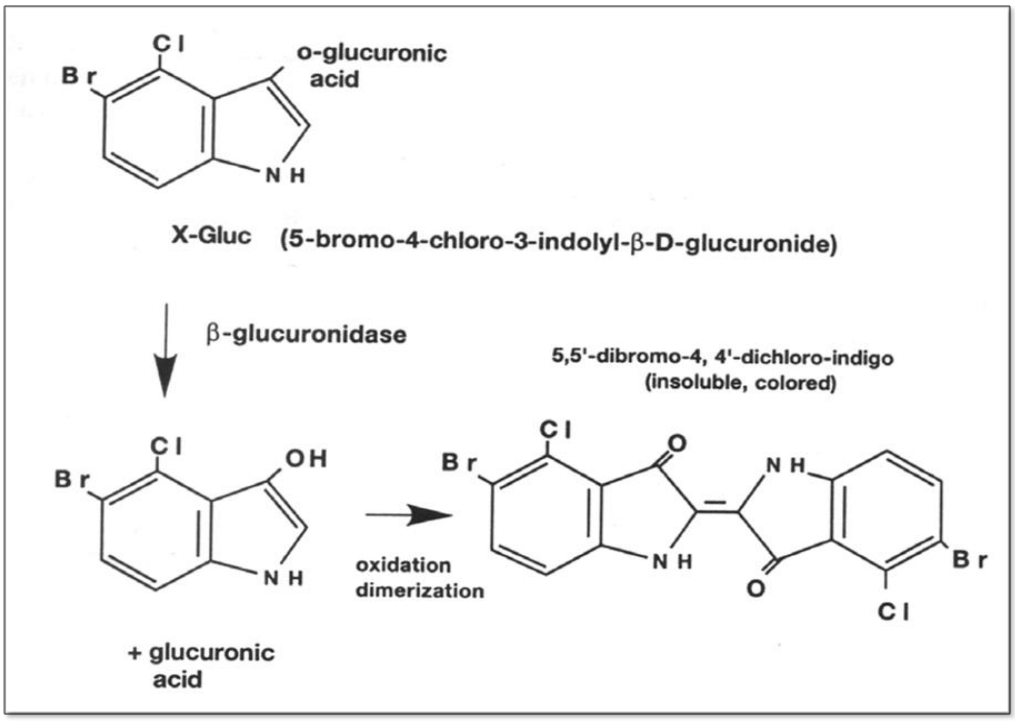


Fig 9: Action of β- glucuronidase on X-Gluc X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), a substrate of β- glucuronidase, is cleaved to produce glucuronic acid and chloro-bromoindigo. When oxidized, chloro-bromoindigo dimerizes to produce the insoluble blue precipitate dichloro-dibromoindigo.

4.2.1.2 Selectable marker genes

To test the effectiveness of kanamycin in selecting transformants, 5-d old cotyledonary petiole explants were cultured on regeneration medium, supplemented with kanamycin (0, 5, 10, 15, 20 mg l^{-1}). After 4- 5 weeks, the callus formation and formation of subsequent adventitious shoots were observed.

Sterilsed seeds were inoculated on MS and MS supplemented with kanamycin (10- 300 mg l^{-1}). After one month of culture the seedlings were observed for their response to *in vitro* culture.

4.2.2 Preparation of competent cells of *Agrobacterium*

A loop full of parental *Agrobacterium* culture was inoculated in LB medium with appropriate antibiotics and was incubated on a rotary shaker at 28°C and 200 rpm for 24 hrs. When the O.D reached 0.5-0.8 at 260 nm, the culture was placed on ice. The bacterial suspension was dispensed into pre-chilled (to 4°C) micro tubes under sterile conditions and the cells were collected at the bottom of the tubes by centrifuging at 4°C and 5000 rpm for 3 minutes. The supernatant was discarded and 0.5 ml of 0.1M $CaCl_2$ was then added under sterile conditions and centrifuged as above. The pellet was again re-suspended in chilled 0.1M $CaCl_2$ .These competent cells were quickly frozen in liquid nitrogen and stored at -70°C after adding 50% sterile glycerol/ 0.075% DMSO, or used immediately after keeping the cells on ice for 30 minutes.

4.2.3 Transformation of *Agrobacterium* cells (Holsters et al., 1978)

To 100 µl of competent cells, 50 to 100 ng of the plasmid with desired gene was added. Cold and heat shocks were given by dipping the tubes in liquid nitrogen for 1- 2 minutes and immediately transferring them to 37°C in water bath for 5 minutes. After heat shock, 0.5 ml of LB medium was added and the tubes were incubated in a rotary shaker at 28°C at 200 rpm for 4-5 hrs. The cells were then spread evenly on LA plates containing appropriate antibiotics and were incubated at 28°C for 48-72 hrs for the appearance of transformed colonies. The plates were stored at 4°C in a refrigerator, where the cells stayed viable for two months. Plasmid DNA was isolated from the colonies to confirm the transformation. For long-term storage of the transformed cells, liquid cultures of the cells were quickly frozen in liquid nitrogen and stored at -70°C after adding sterile 50% glycerol.

4.2.4 Plasmid isolation from Agrobacterium

After transformation, a single colony of *Agrobacterium* cells was inoculated in 10 ml of LB medium containing appropriate antibiotics and incubated overnight on a rotary shaker at 28°C and 200 rpm. The overnight grown culture was transferred to micro tubes and was centrifuged at 5000 rpm for 1 minute. The supernatant was

discarded and the cells were resuspended in 0.2 ml of ice-cold solution I [4.0 mg l^{-1} lysosyme, 50 mM Glucose, 10 mM EDTA, 25 mM Tris HCl (pH 8.0)] and the tubes were incubated at RT for 10 minutes. To this suspension, 0.4 ml of freshly prepared Solution II [1% SDS, 0.2 N NaOH] was added. After mixing the contents thoroughly, the tubes were incubated in ice for 5 min. The contents were mixed for few seconds or till the suspension became viscous. To this viscous lysate, 300 µl of Solution III (5M potassium acetate, pH 5.4) was added and was mixed well and incubated in ice for 5 min. The tubes were centrifuged at 5000 rpm for 3 minutes. The supernatant was taken in a fresh tube and equal volume of ice cold isopropanol was added to the tube. The content was mixed thoroughly by inversion and was incubated at -70 ^{o}C for 15 minutes. After incubation, the tubes were centrifuged at 5000 rpm for 3 minutes and the supernatant was discarded. The precipitated DNA was washed with 1 ml of 70% ethanol and the tubes were centrifuged at 5000 rpm for 1 minute. The supernatant was decanted and the tubes were allowed to stand in inverted position until the supernatant drained off completely. The pellet was air-dried. The dried DNA pellet was dissolved in 50 µl sterile mili Q-water and was stored at -20^{o}C.

4.2.5 Optimisation of parameters for regeneration amenable transformation

The parameters like the strain of the *Agrobacterium* harboring the p35S*GUS*INT binary plasmid vector, cell density of bacterial culture (O.D600), mode of infection (vacuum infiltration/suspension) were standardized in transient transformation studies. In some cultures, keeping other parameters constant, the phenolic compound, acetosyringone (AS), which induces the *Agrobacterium* virulence genes, was added at a concentration of 100 µM to the bacterial suspensions prior to use and all the cultures were compared for transient transformation efficiency.

4.2.6 Histochemical assay of GUS

To check the explants, whether it is suitable for transformation or not, preliminary transformation was done using 5-d old cotyledonary petiole explants through *Agrobacterium* strains harboring the binary vector pCAMBIA2301 with *gus* reporter gene driven by 35S promoter. Transient GUS was histochemically assayed after 2-

days of co-cultivation by staining the cotyledonary petioles in a buffer containing X-GLUC (Jefferson et al., 1987). Briefly, 10 explants in 3 replicates were incubated in X-GLUC overnight at room temperature in dark and washed with methanol for removing chlorophyll content. The stained tissues were visualized under a stereo microscope and scored. It was observed that the cut ends of the transformed explants showing blue coloration indicated the transient expression of *gus* in all the superficial cells and it was absent in the non-transformed control explants.

4.2.7 *Agrobacterium* mediated transformation of cotyledonary petioles

4.2.7.1 Agrobacterium culture preparation for transformation

The *A. tumefaciens* strains (LBA4404 and EHA105) were transformed by freeze-thaw method with the binary vector pCAMBIA 2301 that contains the reporter gene *gusA/uid-A* and plant selection marker *npt*II (that confers kanamycin resistance), both under the control of CaMV 35S promoter. The *gusA* gene, which encodes β-glucuronidase, is interrupted by an intron sequence of castor seed catalase gene in its T-DNA region. This intron is spliced only during eukaryotic expression. The transformed colonies were selected on solid Luria Agar medium containing 25 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin. Liquid cultures were initiated by inoculating single colony of the bacterial strain harboring this plasmid in YEM medium containing antibiotics. The cultures were grown overnight on a rotary shaker at 28°C at 200 rpm. Bacterial concentration was adjusted to desired O.D in a spectrophotometer at 600 nm. The bacterial suspension was centrifuged at 5,000 rpm for 10 min at 4°C. The pellet was resuspended in sterile double distilled water and was used to infect the explants. The cotyledonary petioles were suspended or vacuum infiltrated in the bacterial suspension for 15 min, blotted and cultured on solid basal medium. The cultures were co-cultivated in dark for 2-days and later assayed for GUS.

4.2.7.2 Transformation procedure:

The most regenerative medium (0.5 mg l^{-1} NAA and 0.5 mg l^{-1} BAP) and the explants (cotyledonary petioles) were used in all the transformation experiments. 5-d old

cotyledonary petioles were excised from the *in vitro* grown seedlings and vacuum infiltrated for 10 min. Prior to re-suspension of explants, 100µM acetosyringone was added to the bacterial suspension. The bacterial suspension was blot dried to avoid overgrowth of bacteria over the explants. After two days, the explants were transferred to the regeneration medium containing 250 mg l^{-1} cefotaxime for inactivating the bacteria. The number of shoot buds developed and remained green after transformation was far less than that obtained in normal regeneration trials. On cefotaxime containing medium, the explants, remained green and callused little and started developing shoot buds from the cut ends of the petiole. Within 7 -10 days after transferring to cefotaxime containing medium, the explants started developing shoot buds. Once the shoot bud induction started, the explants were subjected to selection pressure to discriminate between the transformed and non-transformed shoot buds. The green shoots were separated and sub-cultured to rooting medium (MS) without selection. Avoiding kanamycin in the rooting media was essential as there was inhibition of roots in the presence of kanamycin. After 5-6 d, emergence of roots could be noticed and the shoots were transferred to rooting medium along with kanamycin (10 mg l^{-1}). The plantlets obtained after 10 days were hardened, acclimatized and transferred to the green house.

4.2.8 DNA extraction from leaf tissues (miniprep):

CTAB method (Murray and Thompson, 1980; Doyle and Doyle, 1990): DNA isolation was done from the second leaf from the shoot tip of young plants. The leaves were freshly collected or frozen in liquid nitrogen and stored at -70°C. The leaf tissue (100-500 mg) was ground using motor and pestle, in liquid nitrogen, to a fine powder along with a pinch of PVPP (Polyvinyl Polypyrrolidone). Warm (65°C) CTAB (Cetyl/ Hexadecyltrimethyl Ammonium Bromide) extraction buffer (1.0 ml) [2% CTAB, 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 2% β-mercaptoethanol (β-merc)] was taken in 2.0 ml micro tubes and 200 mg of the ground tissue was transferred to the tube, and mixed well to suspend the powder uniformly by repeated inversion of the tubes. The tubes were then incubated at 65°C for 1 hr with intermittent mixing. After incubation, 0.5 ml of Chloroform: Isoamyl

alcohol (24:1) mixture was added and the contents were mixed thoroughly by inversion. The two phases were separated by centrifugation at 12,000 rpm for 15 minutes. The upper aqueous layer was taken in a fresh 2.0 ml tube without disturbing the middle and lower layers. The nucleic acid content was precipitated from the aqueous phase by mixing well by inversion after adding an equal volume of isopropyl alcohol and incubating the tubes at -20^{o}C for a minimum of 30 minutes. After the cold treatment, the tubes were centrifuged at 4^{o}C at 12,000 rpm for 15 minutes to sediment the nucleic acid pellet. The supernatent was decanted completely and 1.0 ml of 70% ethanol was added, and incubated for 5 minutes at RT. The tubes were centrifuged at 12,000 rpm 3-5 minutes and ethanol was decanted. The pellet was air-dried and dissolved in 500 µl sterile milli-Q water was added.

4.2.8.1 Purification of genomic DNA

The genomic DNA isolated from the tissue was purified from contaminating RNA and protein in the purification procedure. This was necessary if the DNA was to be used for molecular analysis. To the dissolved nucleic acids, 2-3 µl of RNase A from a stock of 10 mg ml^{-1} solution was added and the tubes were incubated at 37^{o}C in a water bath for 1-2 hrs. Equal volume (here 500 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and mixed carefully by inversion. When the two phases got mixed properly, the tubes were centrifuged at 12,000 rpm for 15 minutes. The upper aqueous layer was removed to a fresh tube without disturbing the middle protein and lower phenol phases. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed thoroughly and centrifugation was repeated.

4.2.8.2 DNA Precipitation

The upper aqueous layer was taken in a separate tube and two volumes of isopropanol was added to it. The content was mixed well by inverting the tubes 5 to 6 times and was incubated at -20^{o}C for a minimum of 30 minutes. After incubation, the tubes were centrifuged at 12,000 rpm for 10 minutes. The solution was decanted and the pellet was rinsed with 70% ethanol as explained earlier. The final

pellet was air dried and suspended in a minimum volume (20 to 50 µl according to the pellet size) of sterile milli-Q water or TE buffer. The isolated DNA was stored either at 4°C (short term) or -20°C (long term).

4.2.8.3 Quantification of DNA

An aliquot (5.0 µl) of the solubilized DNA was mixed with 1.0 ml of sterile milli-Q water and measured A_{260} and A_{280} of the resulting solution. The DNA content was calculated assuming that one A_{260} unit equals 50 µg of double stranded DNA/ml.

4.2.9 *In planta* genetic transformation of seedlings by *Agrobacterium* infection of apical meristems

5-d old, *in vitro* grown seedlings were used as explants in transformation experiments. One of the cotyledons is detached from the seedling carefully using a sharp scalpel blade so that the meristem does not get damaged on removal. Overnight grown *Agrobacterium* (LBA 4404) harboring the plasmid pCAMBIA 2300 with *TvD₁* **(Fig 10)** (O.D 0.5) was resuspended in half strength MS and taken in a 90 mm Petri plate. Seedlings with one cotyledon attached avoiding the hypocotyl and radicle were suspended in the bacterial culture and vacuum infiltrated for 10 min.

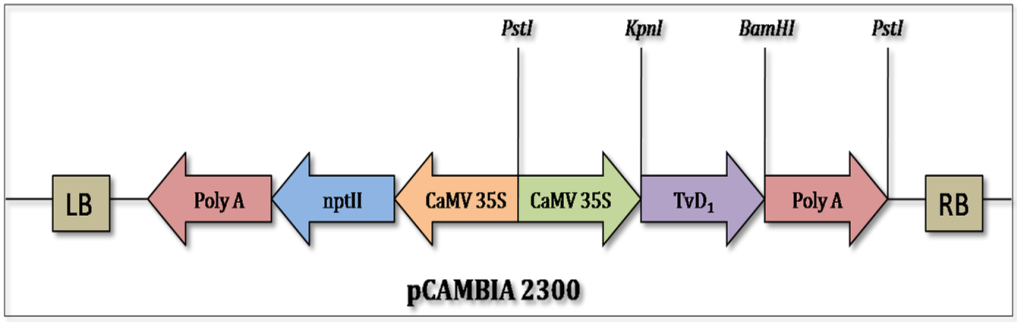


Fig 10: Partial map of vector pCAMBIA 2300 harboring TvD_1 gene cassette

They were blot dried and cultured on MS medium in culture bottles in dark at culture room conditions for 2 days. Later they were transferred to light and maintained for 10 – 12 days till they developed true leaves. The seedlings were washed to remove excess medium from the roots and transferred to soil-vermiculite mixture (1:3) and later transferred to green house for subsequent growth.

4.2.10 Molecular analysis using PCR amplification of *nptII* and *TvD1* gene

Molecular characterization of the transformants was carried out by PCR for the confirmation of the presence of introduced genes. Genomic DNA was isolated from young leaves using CTAB method (Doyle and Doyle, 1990). PCR amplification was carried out with gene specific primers for *nptII* and *TvD_1* using genomic DNA from putative transformed plants, non-transformed control plants (negative control) and vector plasmid (pCAMBIA 2300 harboring *TvD_1* gene and *nptII)* (positive control). To eliminate the presence of native gene amplification, forward primer of 35S promoter and reverse primer of the gene were used in the PCR reaction set up.

Primers used in the experiment were as follows:

35S forward: 5' – GCT CCT ACA AAT GCC ATC A- 3'

nptII reverse: 5'- ATC GGG AGC GGC GAT ACC GTA-3'

TvD_1 reverse: 5'- GGG ATC CTT TAA CAT CTT TTA GTA CAC CA-3'

Each reaction was carried out in a 50 µl (total volume) reaction mixture consisting of 1X PCR buffer, 0.2 mM each dNTPs, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.5 units of Taq DNA polymerase and 10 to 20 ng plant DNA from putative transgenic and control non-transgenic plants or plasmids. Amplification was carried out in a thermal cycler (Eppendorf® Master Cycler) under following conditions:

94 ^{o}C for 3 minutes initial denaturation,

94 ^{o}C for 1 minute denaturation,

58 ^{o}C for 55 seconds annealing,

72 ^{o}C for 1 minute elongation,

10 minutes at 72 ^{o}C final extension

35 cycles of amplification (steps 2-4).

Lid temperature was set at 105 ^{o}C before the reaction was initiated. Amplified DNA fragments were electrophoresed on 0.8% agarose gel stained with EtBr. Gels were photographed under UV-light in a Gel-Documentation System (LTF Labortechnik, Germany).

4.2.11 *In planta* genetic transformation of mature embryos by *Agrobacterium* carrying p35SGUSINT vector

Seeds (*C. capsularis* cv. JRC 321) were surface sterilized as described in chapter 3 and incubated overnight in sterile distilled water. The embryos were isolated from the seeds carefully along with attached cotyledons and were dessicated for 4 hrs by leaving them in the air flow chamber. Overnight grown *Agrobacterium* culture (LBA 4404) was pelleted and resuspended in sterile water (O.D-0.3). The bacterial suspension was added drop by drop over the dessicated embryos and later transferred to eppendorf tubes containing a pinch of sand. The embryos were left in a rotary shaker at room temperature for two days. Later the embryos were blot dried and inoculated on MS solid medium for germination in dark. The explants were tested for the presence of X-GLUC activity.

4.2.11.1 GUS histochemical assay of mature embryos

GUS assay was performed according to Jefferson et al., (1987). In brief, the embryo explants were incubated in X-GLUC overnight at room temperature in dark and chlorophyll removed with methanol. The stained tissues are visualized by naked eye or under a microscope and scored.

4.3 RESULTS AND DISCUSSION

4.3.1 Marker genes for plant transformation

The production of transgenic plants involves the delivery of a foreign gene of interest and a selectable marker gene that enables the selection of transformed cells, calluses and embryos. This is necessary because of the low transformation frequencies, which result in a few transformed cells among the bulk of untransformed ones. In the selection systems, the selectable marker genes encode either herbicide tolerance or resistance to antibiotics. The most widely used selectable marker gene is the neomycin phosphotransferase gene, which confers resistance to aminoglycoside type antibiotics such as kanamycin.

The *bar* gene was cloned from *Streptomyces hygroscopicus*, an organism which produces the tripeptide bialaphos as a secondary metabolite. Bialaphos contains phosphinothricin, an analogue of glutamate which is an inhibitor of glutamine synthetase (Thompson et al., 1987). The *bar* gene confers resistance to an antibiotic called bialaphos (Murakami et al., 1986). Bialaphos is now being used in agriculture as a non-selective herbicide. It is a tripeptide which is composed of two L-alanine residues and an analogue of glutamic acid known as phosphinothricin (PPT). Basta® is a unique broad spectrum, non-selective herbicide that provides excellent crop safety with a relatively low hazard to the operator. Basta is registered for use in a wide range of crops including avocado, banana, citrus, mango, lychee, pawpaw, rambutan, tree nuts and stonefruit. The selectable bar gene encodes the enzyme phosphinothricin acetyl transferase (PAT) that inactivates Phosphinothricin (PPT), the active ingredient of the herbicide by acetylation and *bar* gene has proven to be a useful selectable and screenable marker for the transformation.

4.3.1.1 Response of explants on kanamycin as plant selectable marker

5-d old Cotyledonary petioles were cultured in the presence of kanamycin at various concentrations (2 – 20 mgl^{-1}). The explants were sensitive to 20 mgl^{-1} concentration of kanamycin that inhibited shoot formation. Sarker et al., (2008) used 100 mgl^{-1} kanamycin in the cultures but did not obtain transgenic plants in their studies. They reportedly obtained green shoots on the selection medium supplemented with 100 mgl^{-1} kanamycin but those shoots did not survive to develop into plantlets. The selection regime followed by the group might not be favorable for jute explants as in our experiments we found tissues were sensitive to kanamycin by becoming necrotic at 20 mgl^{-1} concentration **(Fig. 11)**. Only 15 mgl^{-1} kanamycin was sufficient to select transformed and the untransformed tissues. Also during rooting, inclusion of kanamycin after a week culture proved beneficial. Those explants that developed few shoot buds bleached at this concentration. Thus, 15 mgl^{-1} kanamycin was considered for all further experiments in our studies. It is also likely that genotypic differences existed for enduring kanamycin in the culture medium.

Fig 11: Effect of kanamycin on cotyledonary petioles (a) explants on 15 mgl^{-1} kanamycin having healthy and bleached shoots **(b)** explants completely bleached on 20 mgl^{-1} kanamycin.

Seed germination appeared to be more resistant to higher levels of kanamycin in the germination medium as even 300 mgl^{-1} concentration did not inhibit seed germination even in control plants. Thus selection of seedlings on kanamycin did

not appear to be an effective approach. Hence alternate selectable markers like gluphosinate (Basta) might be used as it can be sprayed or painted over the leaves with effective visible symptoms.

4.3.1.2 Response of leaf discs on gluphosinate as plant selectable marker

Control leaf discs were subjected to gradient of the Basta® selection. The control leaf discs after 72 h remained green where as at 0.2 % concentration most of the discs showed signs of necrosis **(Fig. 12)**. At a concentration of 0.1 %, the leaf discs remained pale green. Thus, basta could be used to select jute plants at the concentration of 0.2 %.

Fig 12: Comparison between Basta® concentrations on the control leaves

When basta is used for selecting plants, the untransformed leaves became necrotic and turned blackish brown in color due to the absence of gene and the explants from putative transformed plants remain green. Hence it is easy and fast to select the transformed and the untransformed plants from large populations.

4.3.2 Response of explants towards Agrobacterium infection upon various parameters based on *GUS* assay

Optimal conditions for transformation based on transient GUS activity that represents early infection events were identified using *Agrobacterium* strain harboring binary vector p35S*GUS* INT. In the preliminary examinations, the ability of the strains EHA105 and LBA4404 to transfer genes was compared by observing transient GUS activity. Blue color patches were observed three days after infection on the petioles. In contrast, untransformed controls did not show any blue staining/spots indicating that there was no background gus like activity in white jute **(Fig. 13)**.GUS activity in tissues exposed to bacteria could be attributed to the actual expression of the *gusA* gene, since it requires the removal of catalase intron, which interrupts the *gusA* sequence during RNA processing by the eukaryotic cells (Ohta et al., 1990) and the prokaryotic system machinery does not exhibit this capability. Multiple factors appeared to play a role in efficient T-DNA transfer and transformation of jute cotyledonary petiole explants. The influence of *Agrobacterium* strain, bacterial concentration, acetosyringone and mode of cocultivation were examined.

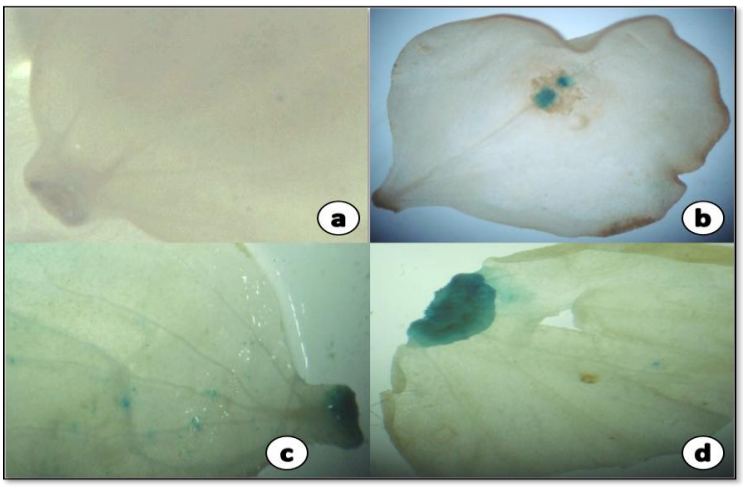
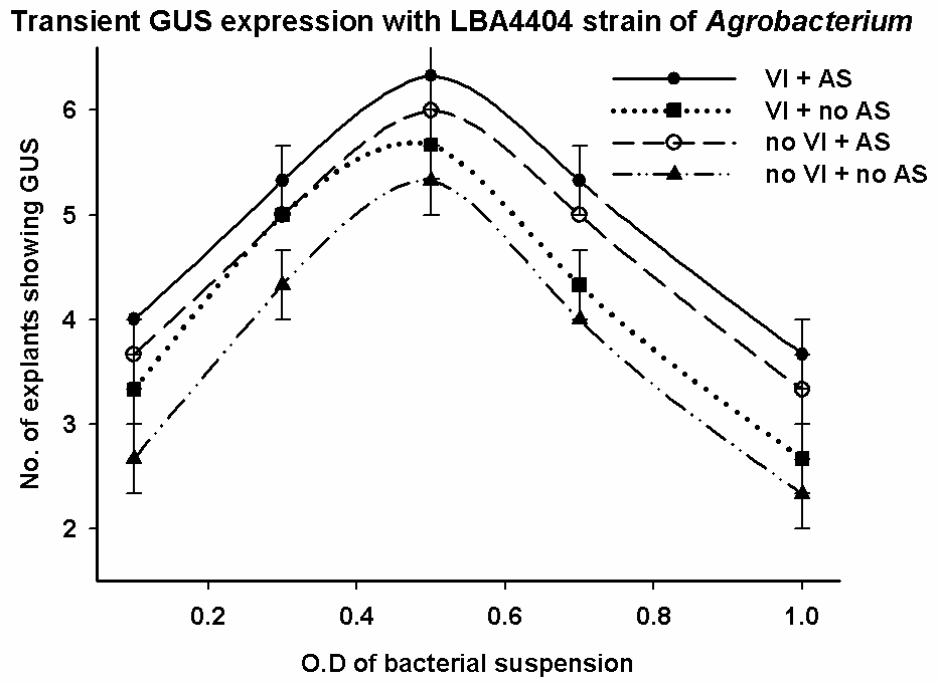


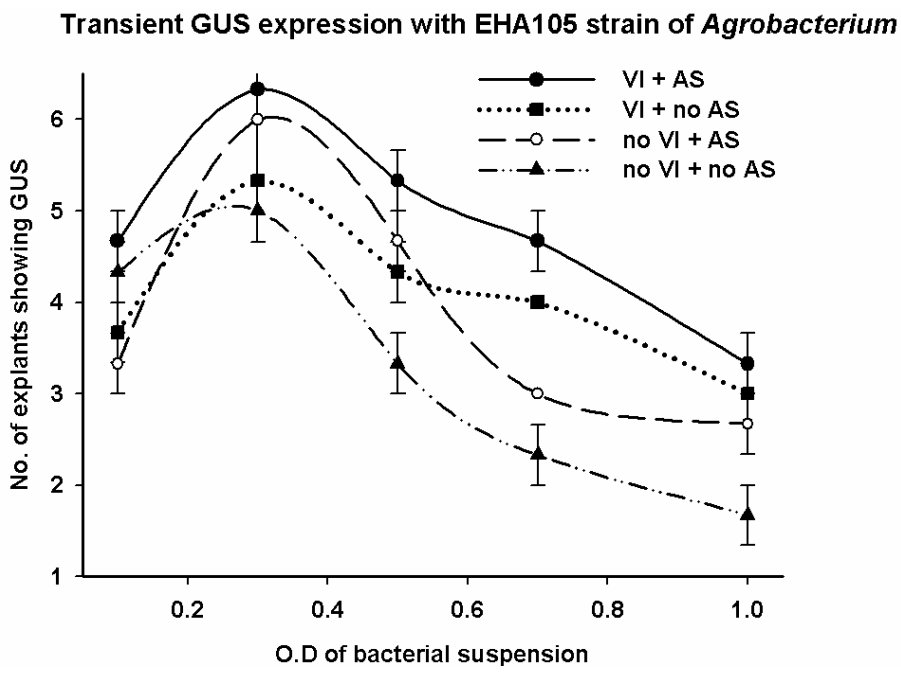
Fig. 13 β-glucuronidase (GUS) transient assay of cotyledonary petioles of jute (10×) (a) Control explant. **(b)** Untransformed explant showing GUS expression on the cotyledonary lamina. **(c)** Transformed explant showing GUS expression at the cut end of the CP. **(d)** GUS expression showing large blue patches at the cut surface of the CP.

4.3.2.1 Effect on addition of acetosyringone

Acetosyringone (AS) is known to enhance the transfer of TDNA from *Agrobacterium* to plant cells in many plants like cotton (Sunilkumar and Rathore 2001), kenaf (Srivatanakul et al., 2001), rice (Asaduzzaman et al., 2008) etc. The primary step in genetic transformation is the attachment of *Agrobacterium* to the host plant. This is facilitated by the genes present on the bacterial chromosome. Compounds like AS are known to induce *Agrobacterium* virulence genes that help in transfer of T-DNA to the host plant genome at the site of injury. In many cases, AS is known to induce expression of vir genes, which is necessary for the generation of T strands and their transfer to the plant cells. We tested the effect of AS by assaying the transient GUS activity produced in cotyledonary petioles upon *Agrobacterium* infection. We observed that the addition of 100 µM AS increased the blue color intensity at the petioles **(Fig. 13)**. From Fig. 14, we could observe that the addition of AS promoted gusA expression in both the strains (curve numbered 1 vs 2). This indicates that the vir- inducing compounds such as AS had a positive effect on the T-DNA transfer in white jute.

Fig. 14: Graph illustrating effect of various parameters affecting transformation efficiency by transient GUS assay





4.3.2.2 Use of different bacterial strains and effect of bacterial concentration

Success in *Agrobacterium* mediated transformation is based on interaction between plant and the bacterium. Most of the identified strains of *Agrobacterium* exhibit wide host range and host specificity in attachment to the plant and transfer of the T-DNA.

We chose to use EHA105 or LBA4404 in our experiments. Both the strains, were effective in T-DNA transfer in various plant species. Bacterial concentration plays a major role in efficient plant transformation. An excess of bacteria around the explant causes necrosis that would impair regeneration of the treated explants. Also, a lower bacterial cell density might not be sufficient to deliver the T-DNA and would compromise on the frequency of transformation. Hence, an optimal bacterial concentration must be maintained in the transformation experiments. EHA105, being more virulent than LBA4404, showed intense blue patches of GUS activity **(Fig. 13)** at the cut ends at a lower bacterial concentration of 0.3 as compared with LBA4404 that showed higher GUS activity at 0.5 OD. EHA105, the agropine type strain gave more number of GUS positives with larger and intense blue color than LBA4404 under the same cocultivation conditions. Herein, the extent of GUS expression is higher in EHA105 at bacterial concentration of 0.3 and decreased consequently with an increase in O.D. In contrast, LBA4404 at OD 0.5 showed high frequency of GUS expression, which decreased upon an increase in OD **(Fig.14)**. Sarkar et al., (2008) used a bacterial concentration of 1.0 to 1.72 for transforming petiole attached cotyledons and mature embryos in the local cultivars of Bangladesh. However, we found that higher bacterial concentration resulted in bacterial overgrowth, which could not be controlled with the antibiotics used and caused necrosis of the explants. These results suggest that LBA4404 at O.D of 0.5 and EHA105 at O.D 0.3 showed better transformation efficiency in *C. capsularis* cv. JRC321.

4.3.2.3 Effect of vacuum infiltration on transformation

Technique of vacuum infiltration in plant transformation was used as early as 1993 for *in planta* transformation of *Arabidopsis* (Bechtold et al., 1993). Many crop plants like cotton (Haq et al., 2004), kenaf (Metinee et al., 2001), rice (Asaduzzaman et al., 2008) have been transformed with vacuum infiltration using the *in planta* method of transformation. Vacuum creates a negative pressure in the plant tissues and decreases the air spaces present between the cells facilitating the infiltration of bacterial cells into the intercellular spaces facilitating the relocation of bacteria along with medium into the plant tissues that are to be transformed. In our treatments, some of the explants were vacuum infiltrated for 15 min and some were subjected to suspension for the same time. We observed that prolonged exposure of the explants to vacuum lead to hyperhydricity and necrosis. However, vacuum infiltrating the petioles for 15 min showed an increase in transient transformation frequency, when compared to suspension cultures **(Fig 14)**, showing the beneficial effects of vacuum infiltration in jute transformation.

4.3.3 Transient GUS expression

The highest infection frequency of cotyledonary petioles was obtained with bacterial strains EHA105 (OD 0.3) or LBA4404 (OD 0.5) by subjecting the tissues to vacuum and supplementing with 100 µM AS. This resulted in an increase in large blue areas at the cut end of the petioles indicating enhanced gene transfer. These results establish that *A. tumefaciens* can be employed for stable genetic transformation in cotyledonary petioles of white jute, since plant regeneration from this species is successful.

4.3.4 Evaluation of putative transgenic plants obtained from transformation of cotyledonary petioles

T_0 plants were raised by using the standard regeneration protocol in the presence of selection antibiotic kanamycin 15 mgl^{-1}. The putative transgenic plants were confirmed through PCR using the promoter 35S forward primer and the marker (*nptII*) reverse gene specific primers as per the protocol mentioned in the materials and methods. Genomic DNA was isolated and used for PCR analysis. Amplification at

1 kb specific for *nptII* and 35S promoter (**Fig.15**) is obtained. Out of 19 plants, seven **(Table 8)** were confirmed as positive from the overall experiments, that is three sets of experiments with 120 explants each.

Table 9: Results of Jute transformation using cotyledonary petioles as explants

Expt no.	No. of infected explants	No. of shoots obtained after selection	No. of rooted shoots	No. of plants survived	No. of PCR positive plants
1	120	37	17	4	1
2	120	34	19	7	3
3	120	35	19	8	3

All experiments are done in triplicates with 40 explants in each set.

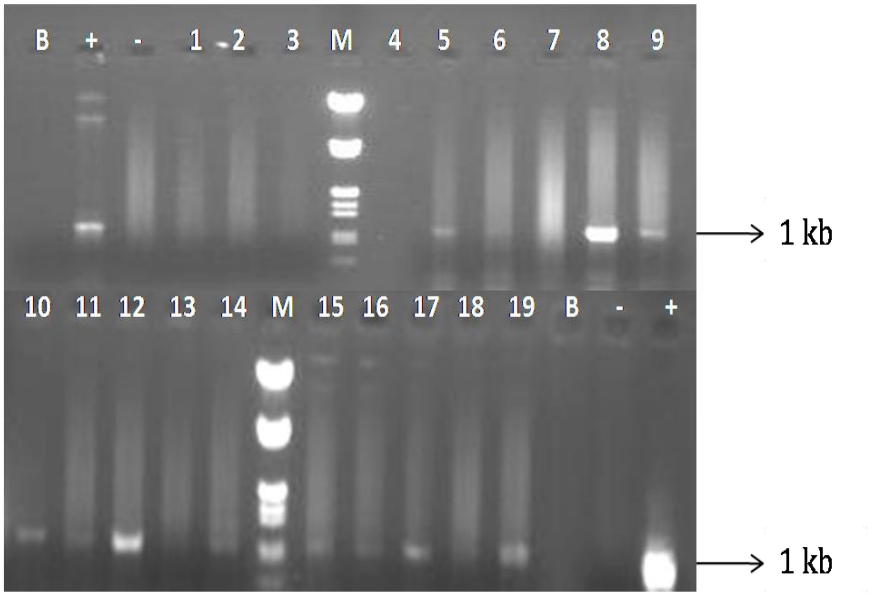


Fig 15: **PCR for the T_0 putative transgenic plants showing the amplification of 1kb *nptII* marker gene** (M) λ EcoRI/HindIIIMarker; (B) BLANK; (+) control plasmid; (-) control plant DNA; 1 – 19: sample DNA

The T_1 plants obtained from germination of seeds from PCR confirmed putative transgenics did not show any amplification of the transgene. The loss of transgene in the subsequent generations remained ambiguous. Uncertainty lies in the fate of introduced DNA getting segregated to the subsequent generations. It might be due to genetic instability or the impotency of the transformed cells to transfer the transgene to further generations. The fate of DNA introduced into plant cells, if could be monitored would provide a basis to answer most of these questions.

4.3.5 Evaluation of putative transgenic plants obtained from transformation of apical meristems

For the genetic transformation of plants, two methods, namely, the *Agrobacterium*-mediated and particle bombardment methods are most widely used (Birch et al. 1997). The former has several advantages over the latter; the former allows for a more stable integration of a defined segment of DNA into the plant genome and generally results in a lower copy number and fewer rearrangements than the latter. Nevertheless, successful transformations that were reported utilized the microparticle bombardment method (Ghosh et al., 2002). The *in planta* transformation method overcomes the disadvantages of the conventional *in vitro Agrobacterium* mediated transformation method. The latter requires sterile condition, is time-consuming. Moreover, somatic mutation or somaclonal variation frequently could occur in plant cells during *in vitro* culture. Also, some plant species are recalcitrant to regeneration. In contrast, *in planta* transformation involves no *in vitro* culture of plant cells or tissue, which is its greatest advantage. Many laboratories have used this technique to transform crops successfully (Bent et al. 2000). Crops like Arabidopsis (Clough and Bent 1998), Wheat (Supartana et al. 2006), Kenaf (Kojima et al. 2000), rice (Supartana et al. 2005). In our experiments, we examined the applicability of *in planta* transformation method to jute.

To confirm the presence of the *TvD_1* gene in plants, PCR amplification was performed on genomic DNA from putative transformants. Using the 35S promoter forward and *TvD_1* gene reverse primers, the PCR products of the expected size (570 bp) were amplified from putative transformants **(Fig. 16)** and no DNA amplification

was detected in the samples from the control plants, thus demonstrating that the plants carried the *TvD_1* gene.

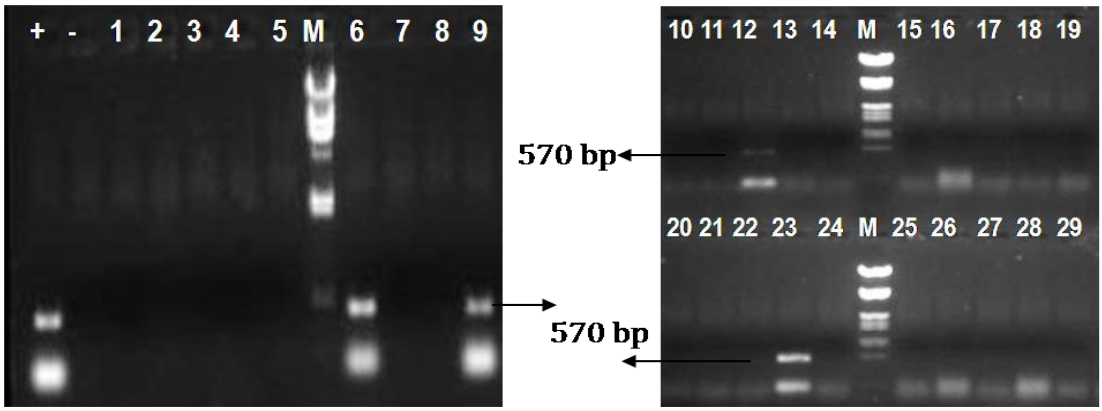


Fig 16: PCR for the T_0 putative transgenic plants showing the amplification of 570kb amplification (M) λ EcoRI/HindIIIMarker; (B) BLANK; (+) control plasmid; (-) control plant DNA; 1 – 29: sample DNA

Though the morphology of the putative transgenics was similar to that of untransformed plants, we observed that there was a reduction in the pod size in all of the transgenic plants **(Fig 17)**. Very few seeds obtained from these pods germinated on MS germination medium.

Fig 17: **Pod size between control and the putative transformed plant**

Interestingly, T_1 plants in this case also did not show any amplification of the transgenes as observed in case of cotyledonary petiole transformation experiments. The lack of transmission of gene to further generations in this crop remained clueless as this is the case observed in any of the methods used for transformations. The fate of the T-DNA in the subsequent generations, if understood can offer better solutions for efficient transformation experiments to be implemented in this recalcitrant crop.

4.3.6 Stable GUS expression observed in various stages of transformed mature embryo explants

The mature embryos after agro-infection showed a positive response towards GUS. This indicated that mature embryos would be a promising source of explant for transformation experiments. The GUS activity was observed in the germinating embryo as well as the seedlings. The mature embryos that were inoculated in water without *Agrobacterium* were used as controls in the experiments. The control explants did not show any gus activity.

GUS activity could be observed throughout the embryos. Young seedlings after 5 days also exhibited blue coloration all through the seedling **(Fig 18)**. This provides a basis that mature embryos could be used for transformation experiments.

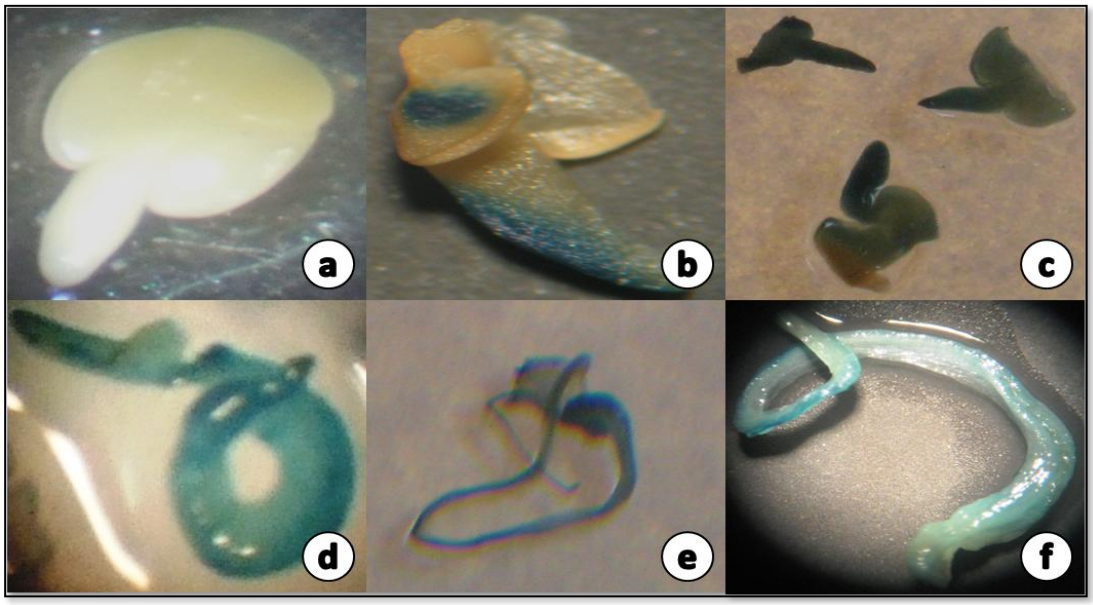


Fig 18: GUS localization in mature embryo explants of jute (a) Control embryo (untransformed) (4x) **(b)** Embryo showing GUS (4x) **(c)** Complete embryos showing GUS (hand held magnifying lens **(d)** Germinating embryo showing GUS in the hypocotyl, cotyledon and the radicle (4x) **(e)** Complete seedling showing GUS (hand held magnifying lens) **(f)** Hypocotyl portion under 4x magnification

Forty eight putative transgenic plants were tested for the integration of the *nptII* gene by PCR amplification. None of the plants showed positive response for the amplification of the *nptII* gene. Closer scrutiny of the GUS positive materials revealed that the blue color was found only in the peripheral tissues. It is possible that the cells that are destined to give rise to germ line cells did not get transformed to result in progenies segregating the transgene(s). Same was the case with meristem tissues. The position of the meristem inside the cotyledons might have made the agro-infection not succeeding. The apical meristem that gives rise to shoot might be concealed within the cotyledons and might not be amenable for agro-infection.

4.4 CONCLUSIONS

The present study provides a rapid and cell culture free method for delivering the transgenes in the germ line tissues of jute. In particular, our findings provide a method of preparing the tissues for transformation and also provide a method for the first time to transform a cell and regenerate the transformed cell into a plant. In the present study, we tried to overcome or at least alleviate one or more of the difficulties or deficiencies associated with the previous findings.

Kanamycin as a selection agent could be used at a concentration of 15 mgl^{-1} and phosphinothricin in the form of Basta could be used at 0.2 % concentration on leaf explants to identify transformed and untransformed from a large population of plants. An improved system of transformation was achieved with the optimized parameters using different explants like cotyledonary petioles, apical meristems of seedlings and mature embryos. A stable integration of the transgene could not be observed which could be due to the complexity of the system and plant material. However, we could provide the basic parameters that can be used for developing improved protocols of transformation for this crop.

Chapter 5

CHAPTER 5

5.1 INTRODUCTION

Jute is a soft, shiny, long, off-white to golden-brown, and cheap natural vegetable fiber that could be spun into strong threads; produced from plants of genus *Corchorus*, belonging to the family *Malvaceae*. This 100% biodegradable and recyclable golden fiber could be successfully blended with variety of other fibers for varied environment friendly and value added end products. *C. capsularis* (white jute) is one of the two cultivated species in the genus. *In vitro* plant regeneration protocols using cotyledon and petiole attached cotyledon explants for different cultivars from both the species are well documented (Abbas et al., 1997; Islam et al., 1982; Seraj et al., 1992; Saha et al., 1999; Huda et al., 2007; Sarker et al., 2007; Pushyami et al., 2011). Jute considered recalcitrant to regeneration and the process being genotype dependent (Naher et al., 2003), different types of explants need to be tried for establishing a repeatable regeneration protocol. Plant regeneration could be achieved from cotyledonary petiole explants till now but protocols for regenerating other explants like hypocotyls and cotyledons were so far not successful.

C. capsularis and *C. olitorius* (tossa jute) have their own unique qualities. While *C. capsularis* could adapt itself to varied growing conditions and has better tolerance to water logging, *C. olitorius* possesses better yield and retting quality. However, genetic diversity in these two species has been narrowed down due to restricted selection from natural populations for quantitative traits. In addition, a strong sexual incompatibility, which causes premature cessation of embryo growth, exists between these two preventing any cross breeding (Ganesan et al., 1957; Kundu et al., 1959). Hence, creating inter-specific hybrids via natural or conventional breeding became very difficult. For the same reason, possibility of improving the fiber has become almost impossible. The only possible way to create hybrids of these species has been recognized as protoplast fusion since long (Das et al., 1981; Arangzeb and Khatun, 1983). Though there were studies on protoplast

culture in cultivated jute species, none of them reported plant regeneration from protoplast derived cultures (Islam et al., 1981; Kumar et al., 1983; Saha and Sen, 1992; and Khatun et al., 2002), the only exception being Saha et al. (2001) who reported a limited success in obtaining embryo like structures from protoplasts.

5.2 MATERIALS AND METHODS

5.2.1 Source of explants

Seeds of the white jute cultivar JRC 698 (obtained from Central Research Institute for Jute and Allied Fibers, Kolkata, India) were surface sterilized with 70% ethanol for 1 min and 0.1 % (*w/v*) mercuric chloride (Himedia, Mumbai, India) for 2 min followed by thorough washing with sterile distilled water.

The 3-d old germinating seedlings on germination medium (MS + 1% sucrose) were transferred to light for further two days for regeneration studies and retained in dark for protoplast studies.

5.2.2 Regeneration experiments

5-d old seedlings were used as explants in regeneration experiments. The cotyledons and the apical bud were excised and separated from the hypocotyl region and hypocotyl explants (1.0 cm long) were cultured on MS medium containing different combinations and concentrations of plant growth regulators (PGRs).

5.2.2.1 Culture media and culture condition

Required PGRs were added to MS basal medium (Murashige and Skoog, 1962) and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Explants were grown on medium solidified with 8.0gl^{-1} agar (Himedia, Mumbai, India) and kept under 16 h photoperiod, illuminated with cool white fluorescent lamps at 28°C. MS basal medium was supplemented with 0.5 - 7.5 mgl^{-1} 6-BAP (6-Benzylaminopurine) in combination with 25 or 50 mgl^{-1} adenine hemi sulfate ($AdSO_4$) for callus induction and subsequent regeneration. The response was carefully monitored and scored.

Calli bearing shoot clumps were sub-cultured to medium containing 0.1mgl^{-1} NAA and 1.0 mgl^{-1} 6-BAP for rapid shoot elongation. The number of elongated shoots was visually examined and the shoots were separated from the clump.

5.2.2.2 Rooting and hardening

Elongated shoots (2–4 cm) were excised and transferred to MS medium for rooting. Individual plantlets were acclimatized in a mixture of soil and vermiculite (1:3) under culture room conditions in plastic cups covered with transparent polythene bags with holes to control humidity by gradually increasing the holes every week for 15 days. Hardened plants were transplanted into pots containing manure and soil (1:2) under green house conditions.

5.2.3 Protoplast isolation

Hypocotyls and cotyledons were transversely chopped in pre-plasmolysis solution **(Table 10)** into fine pieces (0.05 – 1.0 mm thickness) in a 9 cm Petri plate. Protoplasts were isolated in a two step - stationary and gyration digestion process. Following 1h pre-plasmolysis and stationary overnight digestion at 25°C in the enzyme solution **(Table 10)** for cell wall degradation, they were incubated at 27°C on a rotary shaker maintained at 50 rpm for 2h. The resultant mixture was pelleted and purified over sucrose solution (**Table 10**). All incubations were carried in dark at 26±1°C.

5.2.3.1 Purification of the protoplasts

The isolated protoplasts with debris were released by pipetting the enzyme protoplast mixture slowly through a wide bore Pasteur pipette. The enzyme solution along with protoplasts is passed through a 45μm steel filter. Most of the cell debris got filtered at this stage. The filtrate was dispensed in a 10 ml sterile screw cap tube and centrifuged at 2000 rpm for 5 min. At this step the vacuolated protoplasts floated over the solution and formed a band that can be easily removed with a Pasteur pipette and the supernatant was discarded. To the pellet, 10 ml of 20% sucrose was added and centrifuged at 2000 rpm for 10 min again. Protoplasts form a band over the sucrose surface that could be collected in a separate sterile tube using a Pasteur pipette. The protoplasts were washed twice with 35% sea water **(Table 10)**. At this step, protoplasts formed a thick pellet at the bottom of the tube. The protoplasts were resuspended in 4 ml of Kao's and Michayluk (1975)

medium (KM) with PGRs and aliquoted in 6cm petriplates to a final cell density of 1 x 10^5 cells/ml and incubated in dark at 25°C.

5.2.3.2 Protoplast Culture

The protoplasts were cultured in liquid medium containing varied growth regulators. Sustained divisions could be observed in KM medium containing 1.0 mgl^{-1}2,4-D, 0.1 mgl^{-1}NAA and 0.05 mgl^{-1} zeatin. All the growth regulators used in culture were filter sterilized using sterile 0.22 μm cellulose nitrate membrane. Dilutions of the culture media were made following the first mitotic divisions for reducing the osmotic pressure of the culture medium.

Table 10: Solutions and culture medium used for protoplast isolation and culture

Media	Composition	Method of preparation	Step where it is used
Plasmolysis solution	½ MS and 7% (*w/v*) Mannitol	pH adjusted to 5.6 autoclaved	Pre-plasmolysis
Enzyme solution	½ MS + 7 % (*w/v*) Mannitol + 1% (*w/v*)cellulose R10 (Yakult Pharmaceutical Industry Co. Ltd, Tokyo, Japan), 0.05% (*w/v*) Pectolyase Y23 (Seishin Corporation, Tokyo, Japan)	pH adjusted to 5.6 and filter sterilized using 0.2 µm filters (Sartorius)	Isolation of protoplasts
Sucrose Solution	20% (*w/v*) Sucrose + 0.05% (*w/v*) $CaCl_2$	pH adjusted to 5.6 and autoclaved	Protoplast purification
Wash solution	35 % Sea water (major nutrients)	Major salts dissolved in double distilled water and autoclaved	Pelleting and washing of the protoplast pellet
Culture medium	Modified Kao's and Michayluk media (1975) along with plant growth hormones	7.2g/l Sucrose is added and pH adjusted to 5.6 and filter sterilized using 0.2 µm filters (Sartorius)	Protoplasts culture medium

5.3 RESULTS AND DISCUSSION

5.3.1 Regeneration experiments

Different explants were used in the past for in vitro regeneration of *C. capsularis* and so far cotyledons with petiole attached has been the most promising explantfor this recalcitrant crop (Khatun et al., 2007). This crop is genotype dependent and fastidious about the explant type used with respect to tissue regeneration (Naher et al., 2003).

$AdSO_4$ is known to be a potent growth regulator often used in callus induction and plantlet regeneration in many plants. Purine based cytokinins – both natural and synthetic are degraded in plants to adenine and related nucleotides (McGaw et al., 1984; Forsynth and Van Staden 1987). Despite its known activity in the adenine derived compounds (cytokinins), adenine itself is used in tissue cultures for plant regeneration. Adenine as a potent growth promoter, when used along with 6-BAP or kinetin often proved more advantageousin organogenesis of many species like *Vigna* (Ayyasami et al., 2002), jojoba (Hassan et al., 2003), pear (Yancheva et al., 2006) etc.

In the present studies, on the hypocotyl explants, callus initiated within 2-3 weeks of culture on the MS medium supplemented with 6-BAP and adenine hemisulfate. The calli obtained from different treatments could be differentiated into two types by color and texture. Type I calli obtained were soft, watery, non-morphogenic and yellow to brown in color during the early weeks of culture. The calli grew very slowly and upon subculture to medium containing 7.5 mgl^{-1} 6-BAP and 50 mgl^{-1} adenine hemisulfate, this callus showed no signs of development and browning of the explants occurred subsequently. Type II calli cultured on 7.5 mgl^{-1} 6-BAP and 50 mgl^{-1} $AdSO_4$ containing medium were friable, cream to white in color which turned green later. The calli were compact and granular in nature. Rapid shoot proliferation could be observed in medium supplemented with NAA (0.1 mgl^{-1}), 6-BAP (1 mgl^{-1}). GA_3 at 0.5 mgl^{-1} along with 0.1 mgl^{-1} BAP was used for shoot elongation. Hypocotyls cultured on the medium containing 7.5mgl^{-1} BAP & 50 mgl^{-1}

$AdSO_4$ showed a maximum response of 25 % shoot bud induction when compared to other combinations **(Table 11)**.

Table 11: Table showing percentage shoot bud induction in hypocotyl explants in response to 6- BAP and adenine hemisulfate supplemented medium

Sl No.	BAP (mgl^{-1})	AdSO4 (mgl^{-1})	% response
1	0.5	25	Callus and later browned
2		50	Callus but later brown when subcultured
3	2.5	25	10%
4		50	16.6%
5	5.0	25	11.6%
6		50	18.3%
7	7.5	25	6.6%
8		50	25%

*calculated as percentage of the number of explants that gave shoot bud induction

5.3.1.1 Rooting and Hardening

Individual shoots were separated from the shoot clumps and rooted on MS basal medium with 1% Sucrose within ten days. The rooted plantlets when transferred to sterile vermiculite and incubated under culture room conditions, 100% acclimatization was observed. The acclimatized plants from the culture room conditions were carefully transferred to pots filled with soil and manure were let to grow in the green house and they established well **(Fig 19)** and produced viable seeds. All regenerated plants appeared normal with respect to morphology, growth and fruit set.

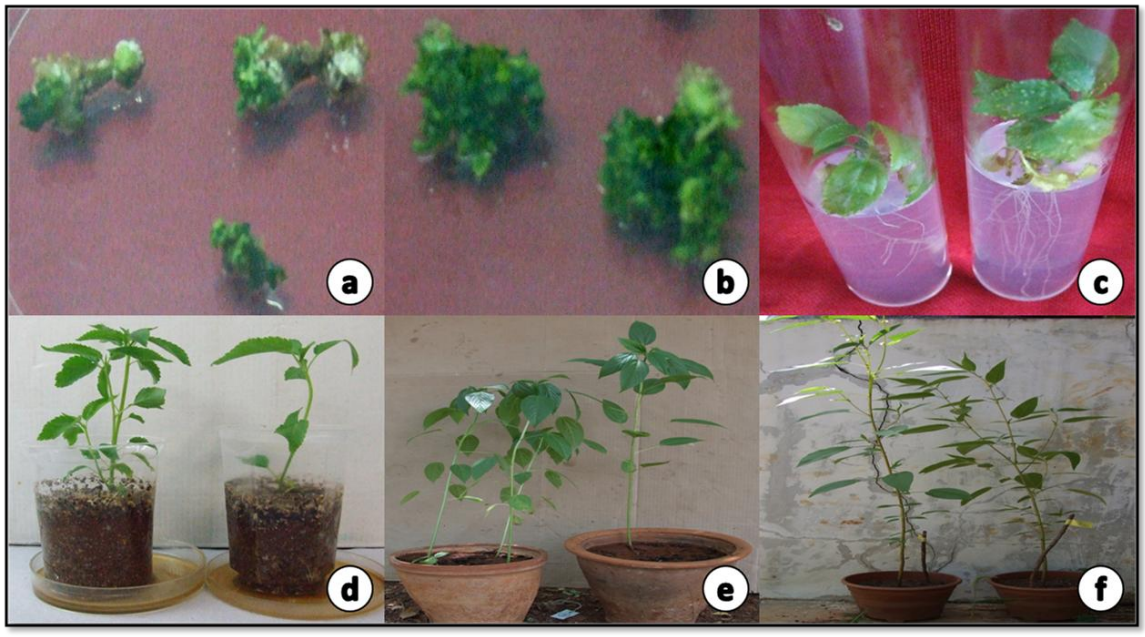


Fig 19: ***In vitro*** **plant regeneration from hypocotyl segments of jute (*Corchorus capsularis* L.*)* cv. JRC 698 (a)** Shoot bud initiation on the hypocotyl. Explant showing white compact granular callus. **(b)** Shoot proliferation from the callus **(c)** *In vitro* rooting of the shoots on MS basal medium **(d)** Acclimatized plants in culture room conditions **(e)** Potted plants in green house conditions **(f)** Regenerated plants that reached flowering stage.

5.3.2 Protoplast isolation

Explant source has always been an important parameter while considering protoplast isolation and regeneration. Different types of explants and sources like leaf mesophyll tissues (Fu et al., 1985; Mei-Lie et al., 1987; Al-Atabee and Power, 1987; Kao and Swartz, 1987; and Castelblanque et al., 2010), callus from different tissues (Sihachkr and Ducreux, 1987; Kransnyanski et al., 1992; and Chabane et al., 2010), cell suspension (Yarrow et al., 1987; He et al., 1992), hypocotyls (Newell and Luu, 1985; Chuong et al., 1987; Wright et al., 1987; Saha and Sen, 1992), and cotyledons (Saha et al., 2001; and Khatun et al., 2002), were used in protoplast isolation in a variety of species including monocots for successful callus induction, regeneration, and somatic embryo development aimed at somatic hybridization and genetic manipulations. Firoozobady et al., (1986) showed that age and growth condition of the donor tissue are very important in achieving cell division and

regeneration of cell wall and cell division. Suitable quantity of tissue must be taken as a starting material for obtaining good number of protoplasts as the final plating density of protoplasts is important for its further development. Since the size of the petiole explant is too small and the callus obtained from it is not enough for protoplast isolations, the other explants that can be used are hypocotyls and cotyledons, which provide good yield of protoplasts from a single experiment.

In our studies for explant standardization, cotyledons, and hypocotyls from dark germinated five day old seedlings were used for protoplast isolation and culture. Mei-Lie et al., (1987) and Hammatt et al., (1988) showed that growing the donor plants in dark for efficient protoplast isolation was important. Hypocotyl sections yielded maximum protoplast density in comparison to cotyledons and protoplast yield was less and of poor quality when younger tissues were used. Hence for further studies only five-day old hypocotyls were utilized. The yield was in an acceptable range, and debris from these preparations could be easily removed by centrifugation. In jute, there are reports of maximum yields from different explants tissues (Saha and Sen, 1992; Saha et al., 2001; Khatun et al., 2002). Hence, the protoplast yield varies considerably depending on various culture conditions and tissue sources. The present results were in accordance with the reports of Saha and Sen (1992), and Saha et al., (2001), however, Khatun et al., (2002) observed that cotyledons were the best source explant for protoplast culture in *C. olitorius*. In contrast to our experiments in which we found 5-day-old seedlings to be better in *C. capsularis*, Khatun et al. (2002) have used 3-day-old seedlings to obtain the optimum results in *C. olitorius*. The use of Cellulase R10 was efficient for degrading the cell walls within a short span of time. Efficiency of Cellulase R10 was also reported by Rethmeier et al. (1991) in *Tomato*, Mathur et al., (1995) in *Arabidopsis*.

One of the major difficulties observed in jute protoplast isolation is the occurrence of high levels of mucilage in the source tissues. Similar observations were also made by Das et al. (1981), Kumar et al. (1983), and Islam (1987). As muscilage content is less in hypocotyls tissues, our experiments were continued with hypocotyl explants. To avoid mucilage secretion, the culture medium and

temperature was altered during seed germination. Similar treatments were made by Khatun et al., (2002) who showed that temperature and growth conditions alter the production of certain compounds in the plant. Use of sea water for washing the explants was previously described by Kumar et al. (1983). Among the three solutions (9% mannitol, CPW salt solution (Frearson et al., 1973) and 35% sea water) used for pelleting the protoplasts in our experiments, it was observed that 35% sea water was very effective as we could get good quantity of protoplasts.

As important as explant source are the combination of enzymes used for cell lysis, protoplast precipitation solution, conditions for plating and components of the medium. The slicing of hypocotyl explants into 1mm size was optimum and yielded good quality protoplasts with one hour pre-treatment in plasmolysis solution. Among a 5% gradient of sucrose solution from 15 to 30% tried, non-vacuolated protoplasts floated and formed a distinct band over 20% sucrose solution (data not shown). High quality non-vacuolated protoplast could be extracted from the solution when 35% sea water was used. Khatun et al. (2002) found that sucrose or percoll solutions were ineffective for protoplast purification. They found 45µm sieve a superior option. Another important factor affecting protoplast yield and division is the pH of the solutions used throughout the experiments. Among various pH values checked, pH 5.6 was found to be best suited for maintaining the integrity and regenerability of the isolated protoplasts (data not given).

5.3.2.1 Protoplast culture

Efficient plating of protoplasts was obtained at normal culture room conditions in Kao's medium. Most of the studies on jute protoplast isolation and culture reported similar observation except for Vaz et al. (1992), who found that high molecular Oxygen atmosphere increased the chances of better plating and plant regeneration capability. For increasing plating efficiency, many groups have added different supplements into the protoplast culture medium or have altered the way the protoplasts were plated. Karamian and Ranjbar (2011) found that the plating efficiency increased upto 5 fold when nurse cells were used in *Muscari*. In the present studies, the plating efficiency was found to be at its optimum when the

isolated protoplasts were cultured in modified Kao's liquid medium. At the plating efficiency of 1 x 10^5 protoplasts /ml of culture, the cell division started within five days of culture, some of the protoplasts regenerated cell walls and started dividing in 24 hours. Sustained divisions were observed after two days in medium containing 1.0 mg/l 2,4-D, 0.1 mg/l NAA, 0.05 mg/l zeatin **(Fig 20)**. The same protocol was tested for 25 other accessions in our laboratory but we could not obtain similar reliable results. This shows that the protoplast isolation and regeneration were purely dependent on the genotype. Similar observations were made by Sihachkr and Ducreux (1987) in sweet potato. Micro-calli were obtained after 60 days of repeated dilutions with medium but regeneration was not obtained even on the most responsive medium standardized for *C. capsularis* (Pushyami et al. 2011) for petiole explants. Though 7.5 mgl^{-1} 6-BAP along with 50 mgl^{-1} $AdSO_4$ was proved beneficial for hypocotyl regeneration in JRC 698, no shoot regeneration could be observed even after 30 days of callus establishment in protoplast cultures. The density of culture media could be a major issue (Chuong et al., 1987; Guang-min et al., 1992). This could be another parameter to be considered while trying regeneration from callus derived from protoplast cultures.

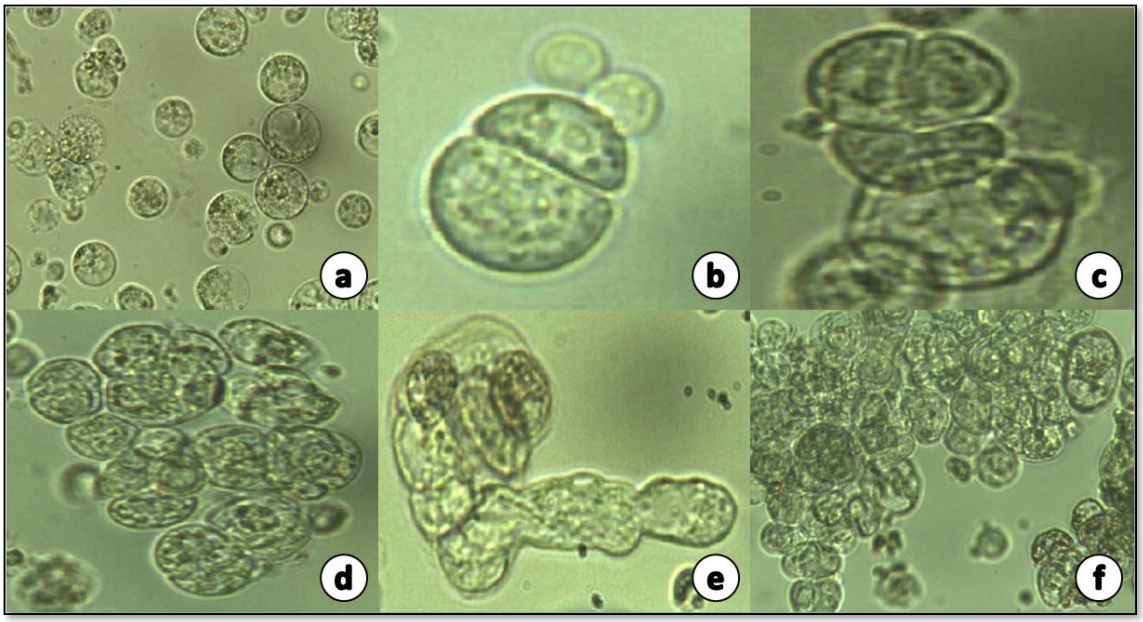


Fig 20: Protoplasts isolated from hypocotyls of Jute (*Corchorus capsularis* L.*)* cv. JRC 698. (a) Hypocotyl protoplasts (20x) **(b)** First divisions (20x) **(c,d)** Second divisions (4- celled stage) (20x) **(e)** Budding and elongation of the cultured protoplasts (20x) **(f)** Dividing cells forming microcalli (20x)

Expression of totipotency in protoplasts is a complex developmental phenomenon and moreover, every step in the protoplast isolation and culture induces stress to the protoplast which affects the cell wall reconstruction, cell elongation and cell cycle re-entrance (Papadakis and Roubelakis-Angelakis, 2002). Further studies must be concentrated on improvement in micro-calli production and standardization of medium for shoot regeneration from callus, so that this protocol could be utilized for further hybridization studies. Reduced anti-oxidant machinery and altered redox homeostatis are other parameters that could be considered when aiming at regeneration from protoplast cultures (Papadakis and Roubelakis-Angelakis, 2002). The increased chance of plating and regeneration capability in presence of high molecular Oxygen atmospheres as found by Vaz et al. (1992) also proves some options. It is also worthwhile to study nurse culture technique as an alternative method of microcalli culture in jute for the exploitation of protoplasts in somatic hybridization and genetic improvement of the crop.

5.4 CONCLUSIONS

Continuous and long history of inbreeding has caused jute cultivars to lose their genetic variability and they are strongly recalcitrant when it comes to in vitro manipulations. The two cultivated jute species, *C. capsularis* and *C. olitorius*, possess the distinct advantages in their own right and need to be hybridized. Exploiting the unique properties of protoplasts could create useful germplasm improvement via somatic hybrids in such sexually incompatible species. For this, a generalized and repeatable regeneration protocol is to be developed in Jute. Culturing of protoplasts poses several problems during cell culture. Mucilage, browning, lack of regenerating lines are few of the difficulties we had come across during the culture process. Although the limited progress gained from this study helps in determining some of the essential factors in isolation and culture of protoplasts in a species of white jute, further study would provide a basis for future work on the development of callus to plant regeneration protocol. The studies and the difficulties reported here could be a guideline for the future researchers to improve their methods.

Chapter 6

CHAPTER 6

6.1 Significance and salient features of the study

The importance of jute (*Corchorus capsularis* L.) as fiber crop was realized by the end of 18th century when it was introduced into world trade through East India Company (Banerjee et al., 1955). Since then, it has become a highly traded commodity next to cotton and generates revenue to our country. Considering the demand for the diversified products of jute, production of finer fiber quality is essential for acceptance of jute products on a commercial scale. The major future thrust area where biotechnology can intervene should focus on crop improvement. A major constraint in the crop improvement is the plant recalcitrance towards tissue regeneration in culture, which is a prerequisite to any genetic modification intervention (Naher et al., 2003).

There are only few reports of plant regeneration in the *capsularis* variety (Saha & Sen 1999 and Sarker et al., 2007). However, plant regeneration in cultures still pose difficulties due to genotype dependence (Naher et al., 2003). With efficient regeneration of explants in cultures, genetic modifications can be performed with the regenerable tissues *in vitro*.

Genetic modification using particle bombardment with successful establishment of plantlets expressing the gene was earlier reported by Ghosh et al., (2002) in the cultivar JRC 321 of *C. capsularis*. Other than being expensive and with known limitations in this method, alternate methods of *Agrobacterium* mediated transformation need to be opted and protocols are to be optimized suitable for this cultivated variety. Reports of Sarker et al., (2008) on *Agrobacterium* mediated genetic transformation of cotyledonary petioles could not revive plantlets in selection medium and hence could not obtain transgenics. The report presents a very good basis for future researchers that the plant is amenable for genetic modifications. The parameters for *Agrobacterium* infection of explants and selection regimes using different selective agents need to be optimized for their application in jute biotechnology.

With the constraints of conventional breeding practices that have been previously reported (Patel & Datta 1960; Islam & Rashid 1960; Swaminathan et al., 1961; Sarker & Hoque 1994), protoplast fusion technique is a powerful tool to produce interspecific hybrids in jute. The reports of Islam et al., (1981); Kumar et al., (1983); Saha & Sen (1992); Saha et al., (2001) produced protoplast cultures. The failure of the protoplasts to divide and form calli or somatic embryos was due to low cell number or other inherent constraints in the methodology. High quality and quantity based method of protoplast isolation method could open avenues in this line of research in jute biotechnology.

In the present study, an overall high regeneration efficiency of jute explants *in vitro* was achieved with minimum modifications in the culture conditions using combination of plant growth regulators. Fertile plants, which were morphologically similar to the cultivated plants, were well established in field conditions.

Parameters were optimized for *Agrobacterium* infection of the explants and evaluated for transient *GUS* expression in the tissues expressing the gene. Further, kanamycin and basta sensitivity assays were performed in order to determine the effective concentration that could completely arrest the growth of untransformed cells.

Efforts were made to apply the *Agrobacterium* mediated method of transformation to the cotyledonary petiole explants of jute using the optimized parameters for agro-infection. We successfully generated fertile putative transgenics confirmed through PCR analysis, but failed to report the same in the subsequent generations. The failure of the gene to segregate in the future generations may be attributed to the genetic instability or the impotency of the plants to segregate the gene in the consecutive generations. The same failure was evidenced by us when alternate approaches of *in planta* method of genetic transformation were also applied.

Technique of protoplast isolation and culture was successfully optimized through which we developed good quality and quantity cultures *in vitro*. Major

drawbacks of tissue digestion, production of poor quantity of cells and excess mucilage in cultures were overcome by modifying the isolation and culture methods. Dividing protoplasts that gave microcalli were generated in less than two months under our laboratory conditions. Since these microcalli showed necrosis on the callus development medium, further parameters need to be optimized for production of embryos or shoots.

6.2 Future prospects

Crop improvement through biotechnology offers a potential for production of better varieties. Jute falls in the category of crop plants that need attention of biotechnologists. Being the largest traded commodity of fibers next to cotton, the main products are its diversified value added products. The trade that generates revenue to the country would rise only if the products are of good quality with respect to the fiber used for making them. Despite its socio-economic importance throughout the world, jute remains to a large extent an underexploited crop.

Since there is a growing demand of jute goods, the quantity not compromising with the quality need to be increased. At this point, biotechnological intervention comes into play where there is a need for generation of elite cultivars that produce finer quality of jute fibers along with increased production. Among the major goals of jute crop improvement are the productivity enhancement with special reference to biotic and abiotic stresses and quality improvement for targeted quality traits.

With our results, it is apparent that the tissue recalcitrance can be overcome in cultures and that the tissues can be subjected to genetic alterations. Other alternate approaches like transforming the mature embryos and screening for a line that has stable gene integration would answer the critical issues that we faced during our studies. It is to be hoped that encouraging progress would be maintained and developed as a continuation of the present study towards redressing the economic growth of this important fiber crop.

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Publications

LIST OF PUBLICATIONS

[1] Pushyami, B., Beena, M.R., Sinha, M.K., & Kirti, P.B. (2011) *In vitro* regeneration and optimization of conditions for *Agrobacterium* mediated transformation in jute, *Corchorus capsularis*. *J. Plant Biochem. Biotechnol. 20*(1), 39-46.

[2] Pushyami, B., Beena, M.R., Sinha, M.K., & Kirti, P.B**.** Studies on regeneration and protoplast culture of hypocotyl explants in jute, *Corchorus capsularis L.* (accepted).

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ORIGINAL ARTICLE

In vitro regeneration and optimization of conditions for *Agrobacterium* mediated transformation in jute, *Corchorus capsularis*

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Abstract Jute is a crop of commercial importance that is widely cultivated for its bast fiber production but susceptible to many diseases that results in major economic loss. New genes can be introduced into this plant through *Agrobacterium* mediated genetic transformation for its genetic improvement, which is dependent on the availability of suitable in vitro techniques. An efficient regeneration system has been developed for in vitro culture of jute (*Corchorus capsularis*) from the distal cut ends of cotyledonary petioles. High frequency shoot regeneration was obtained on Murashige and Skoog (MS) nutrient agar medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 36 g/l sucrose. On transfer to soil, the regenerated plantlets survived and appeared to be morphologically similar to the normal seed-grown plants. They developed pods and set fertile seeds. Histological analysis revealed de novo origin of shoot buds in the in vitro cultured cotyledonary petioles. Parameters affecting transformation were optimized by assaying GUS activity in these regenerable tissues after cocultivation with Agrobacteria. These tissues appear to be susceptible for infection and transformation by *Agrobacterium* carrying *uid* (GUS INT) and *nptII* genes, as well as shoot multiplication. The cells at the cut end of the petioles were found competent to take up the DNA, which was monitored by transient GUS gene expression. EHA105 at 0.3 O.D and LBA4404 at 0.5 O.D were found to be compatible in giving optimal levels of transient GUS expression.

Keywords Transient transformation · GUS · Regeneration · Jute · *Corchorus capsularis* · Cotyledonary petiole · Whole plants

Abbreviations

NAA	α-naphthalene-acetic acid
BAP	6-Benzyl-amino-purine
IAA	Indole-3-acetic acid
TDZ	Thidiazuron
GA_3	Gibberellic acid
2,4-D	2,4-dichlorophenoxy-acetic acid

Introduction

Corchorus capsularis, white jute is widely cultivated over the Gangetic plains of North-eastern India. It is a fiber (bast) yielding cash crop with versatile uses in industry. It is the second cheapest fiber after cotton in production. Aiming at mass propagation and further quality improvement, micropropagation protocols for this species have been tried out in the eighties (Ahmed et al. 1989) followed by the reports on regeneration from aseptically grown seedling explants (Abbas et al. 1997; Khatun et al. 1993a, b; Saha et al. 1999; Naher et al. 2003). However, there was no convincing report in terms of high frequency multiple shoot regeneration and establishment of plants in the field. There are reports of somatic embryos obtained from the protoplast derived calli of *C. capsularis*, which could not be established into plantlets. (Saha and Sen 1992). It was reported earlier that in vitro regeneration in this genus is

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genotype-dependent and the material is recalcitrant to regeneration (Naher et al. 2003). There are reports of regeneration in the Bangladeshi varieties of tossa jute *C. olitorius*) (Khatun et al. 2003; Huda et al. 2007; Khatun et al. 1992). Sarker et al. (2007) reported regeneration in white jute in the local cultivars of Bangladesh. However, the use of the same regeneration protocol was not effective in regenerating Indian genotypes of *C. capsularis* indicating that different cultivars need varied growth regulator combinations. There are no reports on genotype independent regeneration from cotyledonary petioles of *C. capsularis*.

Finer fiber quality is essential for acceptance of jute products on a commercial scale. One of the popular cultivars of *C. capsularis* in India, JRC 321(Sonali) has a fiber fineness about 10 denier than the other released varieties that varied between 16 and 18 denier. Also, this variety can be grown with little soil moisture and can be harvested early.

Many useful characters can be exploited by interspecific crosses between the wild and cultivated species. Due to strong genetic barriers and the inability to produce a viable hybrid, this method did not appear practical. An alternative to overcome this limitation is to introduce suitable new genes through *Agrobacterium*. The reports on the genetic transformation of jute are scanty and inconsistent. There is no report using *Agrobacterium* mediated genetic transformation by multiple shoot regeneration in the local varieties of white jute except an attempt using the biolistic method (Ghosh et al. 2001).

Hence, the present study is undertaken with a popular cultivar of white jute (JRC321) for preliminary transformation and regeneration studies. The susceptibility of cotyledonary petiole explants to various strains of *Agrobacterium* under varied conditions was studied through transient GUS experiments. Our observations are reported in this communication.

Materials and methods

Preparation of seedling explants

Seeds of *C. capsularis,* cv. JRC 321 (obtained from Central Research Institute for Jute and Allied Fibers, Kolkata, India) were surface sterilized with 0.1% (*w*/*v*) mercuric chloride for 1 min followed by rinsing thoroughly 5-6 times with sterile distilled water. Sterilized seeds were germinated on hormone free MS (Murashige and Skoog 1962) medium solidified with agar (0.8%). The seeds were incubated in dark for germination and thereafter maintained at 25±2°C with 16 h light/8 h dark condition in a growth room. The cotyledonary petioles from aseptically grown seedlings were used as explants. While preparing the cotyledonary petiole explants, the pre-existing axillary bud was carefully excluded by cutting the petioles at least 1 mm away from the axil. Further, all those explants that developed a single healthy shoot within 5 days of culture were eliminated to avoid the proliferation of the explants with the pre-existing meristem.

Agrobacterium mediated transformation of cotyledonary petioles

The *A. tumefaciens* strains (LBA4404 and EHA105) were transformed by freeze-thaw method using the binary vector pCAMBIA 2301 that contains the reporter gene *gusA*/*uid-A* and plant selection marker *nptII* (that confers kanamycin resistance), both under the control of CaMV 35S promoter. The *gusA* gene, which encodes β- glucuronidase, is interrupted by an intron sequence of castor seed catalase gene in its T-DNA region. This intron is spliced only during eukaryotic expression. The transformed colonies were selected on solid Luria Agar medium containing 25 mg/l rifampicin and 50 mg/l kanamycin.

Liquid cultures were initiated by inoculating a single colony of the bacterial strains harboring this plasmid in YEM medium containing antibiotics. The cultures were grown overnight on a rotary shaker at 28°C at 200 rpm. Bacterial concentrations were adjusted to desired O.D in a spectrophotometer at 600 nm. The bacterial suspensions were centrifuged at 5,000 rpm for 10 min at 4°C. The pellet was resuspended in sterile double distilled water and is used for infection. The cotyledonary petioles were suspended or vacuum infiltrated in the bacterial suspension for 15 min, blotted and cultured on solid basal medium. The cultures were co-cultivated in dark for 2-days and later assayed for GUS.

The parameters like the strain of the *Agrobacterium* harboring the p35SGUSINT binary plasmid vector, cell density of bacterial culture ($O.D_{600}$), mode of infection (vacuum infiltration/suspension) were standardized in transient transformation studies . In some cultures, keeping other parameters constant, the phenolic compound, acetosyringone (AS), which induces the *Agrobacterium* virulence genes, was added at a concentration of 100 μM to the bacterial suspensions prior to use and all the cultures were compared for transient transformation efficiency.

Culture medium and conditions for plant regeneration

MS basal media were supplemented with plant growth regulators (NAA, BAP) and the pH was adjusted to 5.8 before autoclaving. Various combinations and concentrations of growth regulators were used to study their efficacy in shoot bud induction (Table 1). While culturing, the cut ends of the petioles were inserted into the media. All the cultures were incubated under standard tissue culture conditions as

Table 1 Effect of growth regulators, IAA, NAA, 2,4 D and BAP on hypocotyls and cotyledonary petiole explants of Jute (*C. capsularis*; JRC 321) in shoot bud induction after 4 weeks of incubation[a]

Growth regulators			BAP (mg/l)			
Auxin (mg/l)			0.5	1.0	2.0	4.0
NAA	0.1	H	0[b]	0	0	0
		P	11.66±0.66[c]	9±0.57	8.33±0.33	0
	0.5	H	0	0	0	0
		P	20.33±0.33	16.33±0.33	9.66±0.33	8.66±0.33
IAA	0.1	H	0	0	0	0
		P	0	0	0	0
	0.5	H	0	0	0	0
		P	0	0	0	0
2,4-D	0.1	H	1[d]	1	1	1
		P	1	1	1	1
	0.5	H	1	1	1	1
		P	1	1	1	1

H Hypocotyl

P Cotyledonary petiole

[a] 30 explants in triplicates

[b] Indicates profuse callusing

[c] Data indicate Mean ± SE

[d] No callusing and no response

mentioned earlier. The number of shoot buds produced per regenerating explant was recorded at regular intervals.

During the culture, the samples were periodically fixed in formalin, acetic acid, 70% alcohol (FAA 1:1:18) and then dehydrated through a graded alcohol series, embedded in paraffin and sectioned at 8–10 μ thickness. The tissue sections were stained with Hematoxylin and Eosin for microscopic observations.

All the experiments were repeated four times with 50 explants in each replication.

Rooting and hardening

Regenerated shoots, about 2–3 cm in length were excised and cultured on MS basal medium without growth regulators for root induction. Rooted shoots were hardened under growth room conditions for 15-days in tea cups containing a mixture of soil and vermiculite in the ratio of 1:3 and covered with polythene bags to maintain high humidity. Acclimatized plants were transferred to pots containing soil and were grown to maturity in the green house.

Optimization of the age of seedlings

Age of the seedlings at the time of explanting was studied to analyze its effect on shoot regeneration. Explants from 5 to 11-day-old seedlings were taken from aseptically grown seedlings for their regeneration ability. The effect of vitamin combinations from MS and B5 (Gamborg et al. 1968) media on shoot proliferation was also studied.

Histochemical assay of GUS

Transient GUS was histochemically assayed after 2-days of co-cultivation by staining the cotyledonary petioles in a buffer containing X-GLUC (Jefferson et al. 1987). Briefly, 10 explants in 3 replicates were incubated in X-GLUC overnight at room temperature in dark and washed with methanol for removing chlorophyll content. The stained tissues are visualized under a microscope and scored.

Statistical analyses

All the data were analyzed for analysis of variance (ANOVA) using SPSS package and the treatment means were compared using sigma plot9.0 software. All experiments were carried in a completely randomized design.

Results and discussion

Shoot regeneration and elongation

Multiple shoot regeneration was observed at the distal cut ends of the cotyledonary petiole after 3 weeks of culture on the regeneration medium. Shoot buds appeared after

2 weeks and shoots were visible by 21-days of culture on the medium. A combination of 0.5 mg/l NAA and 0.5 mg/l BAP with 36 g/l sucrose (termed as regeneration medium) promoted 67.8% shoot regeneration frequency with shoot bud differentiation at the distal cut end of the cotyledonary petiole. Shoot buds developed directly from the junction of the petiole and cotyledonary lamina (Figs. 1a–f, 2f). They also developed from the callus produced at the cut ends and were glossy, dark green in nature. Low shoot regeneration efficiency was noticed at lower concentration of NAA with various BAP levels (Table 1). An average of 5-6 shoot buds developed initially. Up-on sequential subculture to 0.3 mg/l and 0.1 mg/l BAP containing medium, the number of shoot buds could be increased to an average of 10 per explant. BAP (0.2 mg/l) in combination with 0.5 mg/l GA_3 was used for rapid shoot elongation. The elongated shoots were of 1–2 cm in length, when they were subcultured for root initiation. When GA_3 alone was added at 1.0 mg/l, necrosis was observed. Shoot elongation was not synchronous in these explants.

In media supplemented with NAA (0.1, 0.5 mg/l) and BAP (0.5, 1.0, 2.0, 4.0 mg/l), white to green fragile callus developed from the explants. Subsequent subculture of this callus did not lead to any shoot bud development. NAA was more suitable for callus formation. However, no difference was observed in callus induction by the addition of 2,4-D regardless of the concentration of BAP used (0.5, 1.0, 2.0, 4.0 mg/l, Table 1). Calli obtained on media containing IAA and BAP were soft and light brown. 2,4-D was also not able to induce shoot regeneration in combination with BAP in the explants of this cultivar (data not shown). The hypocotyl-derived calli on NAA and BAP were green in color with the formation of green nodular structures after 6 weeks of culture. However, these nodular structures did not proliferate further into embryo or shoot-bud like structures. The difficulty of regenerating plantlets from hypocotyl derived calli must be investigated further. These observations indicate that NAA influences callus induction in tissue cultures of *C. capsularis* (JRC 321) and that a combination of NAA and BAP is promising for shoot bud induction in petiole explants. It was observed that the response of regeneration obtained on MS vitamins was better than the composition of B_5 vitamins.

Multiple shoots developed on media supplemented with 0.5 mg/l NAA and 0.5 mg/l BAP only. Data on shoot bud development were collected at regular intervals. The elongated shoots were separated from the shoot clumps and transferred to root induction medium without any growth regulators. Rhizogenesis was observed from the regenerated shoots 2 weeks after transfer of shoots to MS medium.

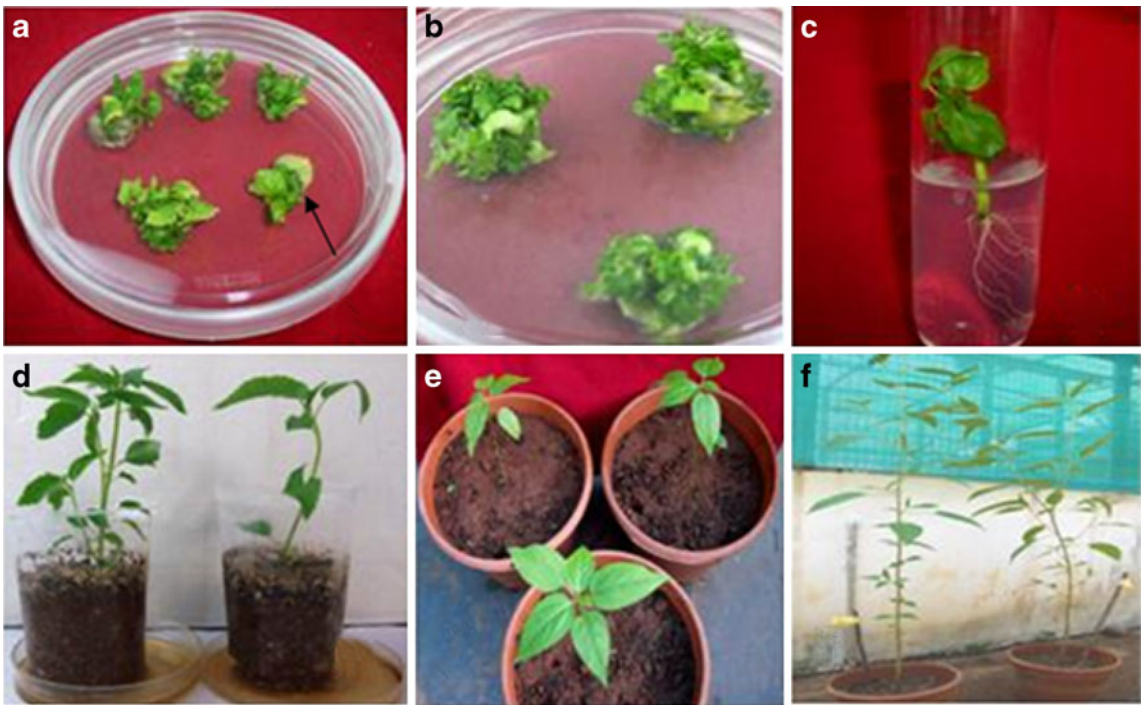

Fig. 1 **a**–**f** Plant regeneration by organogenesis from cotyledonary petiole explants in Jute (*Corchorus capsularis L.).* **a** Origin of shoot buds at the junction of cotyledon and petiole (indicated by an arrow) with intact cotyledon. **b** Proliferation of shoots. **c** Rooting of regenerated shoots. **d** Acclimatized and hardened plantlets. **e** Well rooted plants transferred to green house. **f** Mature plants bearing flowers and pods

Histological analysis

Cross sections of the explants that were fixed at different stages revealed that the regenerated buds developed from the epidermal cells and these cells divided initially followed by the divisions in the inner rows of cells and the stimulated cells beneath developed shoots. The gradual development of shoot buds is shown in Fig. 2(a–f). The developed shoot notch could be clearly observed after 9th day of inoculation. Well developed shoots could be observed by 15th day of the culture.

Effect of age of seedlings

The age of the donor seedlings and regeneration capacity showed an inverse relationship with the percentage of cultures showing shoot regeneration. In all the experiments aimed at testing the effect of the age of explants, the number of explants producing shoot buds declined with the increase in age of the seedlings acting as donors for the petiole explants (Fig. 3). As far as the seedling age is concerned, explants from 5-day old seedlings showed the highest response. Also, we did not notice significant variation between 5 and 7-day-old seedlings. Thereafter, there was a significant decline in shoot bud differentiation with increased age of seedlings acting as explant donors. The *F*-statistics of shoot bud induction showed that the age of the seedling had a significant effect on shoot bud induction .The day-wise performance evaluated by multiple-comparison test indicated that the difference in the mean regeneration frequencies between 5 and 7-day-old explants was not significant. However, the number of shoot buds produced on explants from 5-day old seedlings was significantly higher compared to the explants prepared from 7-day-old seedlings. These observations clearly suggest that the 5-day old seedlings are the best source for generating the cotyledonary petiole explants in *C. capsularis*.

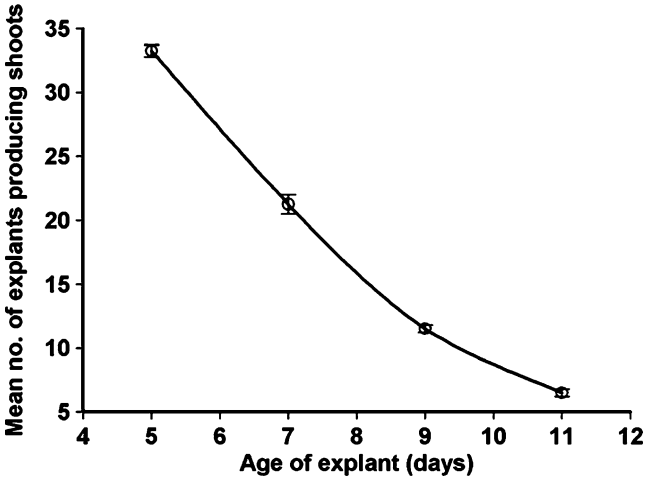

Fig. 3 Graph illustrating the effect of age of seedling with the mean number of explants producing shoots

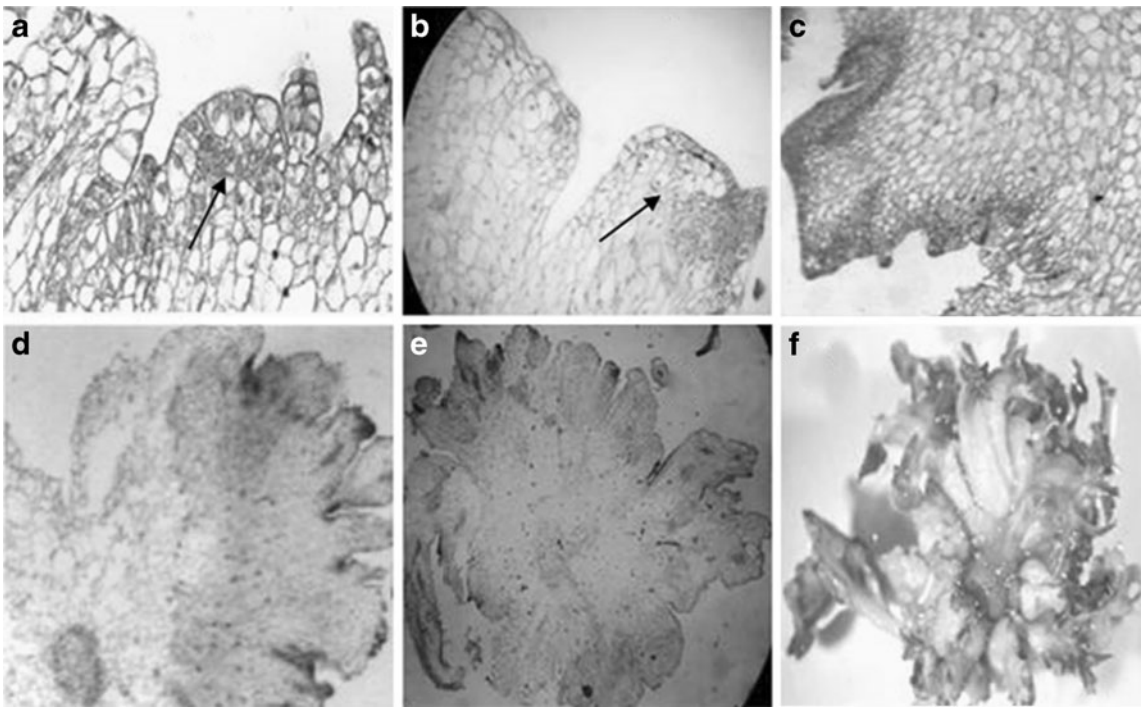

Fig. 2 **a** First divisions of epidermal cells (40×). **b** Further divisions to the inner row leading to a bud-like projection (40×). **c** Well formed shoot bud projection (10×). **d** Shoots formed which are connected to the cambium (10×). **e** Horizontal sectional view of the explant which has multiple buds (10×). **f** Regenerated shoots from the abaxial side of the explant as seen under stereo-zoom microscope

Transient transformation of cotyledonary petioles with p35SGUS INT construct

Optimal conditions for transformation based on transient GUS activity that represents early infection were identified using *Agrobacterium* strain harboring binary vector p35S*GUS* INT. In the preliminary examinations, the ability of the strains EHA105 and LBA4404 to transfer genes was compared by looking for transient GUS activity. Blue color patches were observed three-days after infection on the petioles. In contrast, untransformed controls did not show any blue staining/spots (Fig. 4). GUS activity in tissues exposed to bacteria could be attributed to the actual expression of the *gusA* gene, since it requires the removal of catalase intron, which interrupts the *gusA* sequence during RNA processing by the eukaryotic cells (Ohta et al. 1990). Multiple factors play a role in efficient T-DNA transfer and transformation of jute cotyledonary petiole explants. The influence of *Agrobacterium* strain, bacterial concentration, acetosyringone and mode of cocultivation were examined.

Addition of acetosyringone

Acetosyringone (AS) is known to enhance the transfer of T-DNA from *Agrobacterium* to plant cells in many plants like cotton (Sunilkumar and Rathore 2001), kenaf (Srivatanakul et al. 2001), rice (Asaduzzaman et al. 2008) etc. The primary step in genetic transformation is the attachment of *Agrobacterium* to the host plant. This is facilitated by the genes present on the bacterial chromosome. Compounds like AS are known to induce *Agrobacterium* virulence genes that help in transfer of T-DNA to the host plant genome at the site of injury. In many cases, AS is known to induce expression of *vir* genes, which is necessary for the generation of T strands and their transfer to the plant cells. We tested the effect of AS by assaying the transient GUS activity produced in cotyledonary petioles upon *Agrobacterium* infection. We observed that the addition of 100 μM AS increased the blue color intensity at the petioles (Fig. 4). From Fig. 5, we could observe that the addition of AS promoted *gusA* expression in both the strains (curve numbered 1 vs 2). This indicates that the *vir*- inducing compounds such as AS had a positive effect on the T-DNA transfer in white jute.

Use of different bacterial strains and effect of bacterial concentration

Success in *Agrobacterium* mediated transformation is based on interaction between plant and the bacterium. Most of the identified strains of *Agrobacterium* exhibit wide host range and host specificity in attachment to the plant and transfer of the T-DNA.

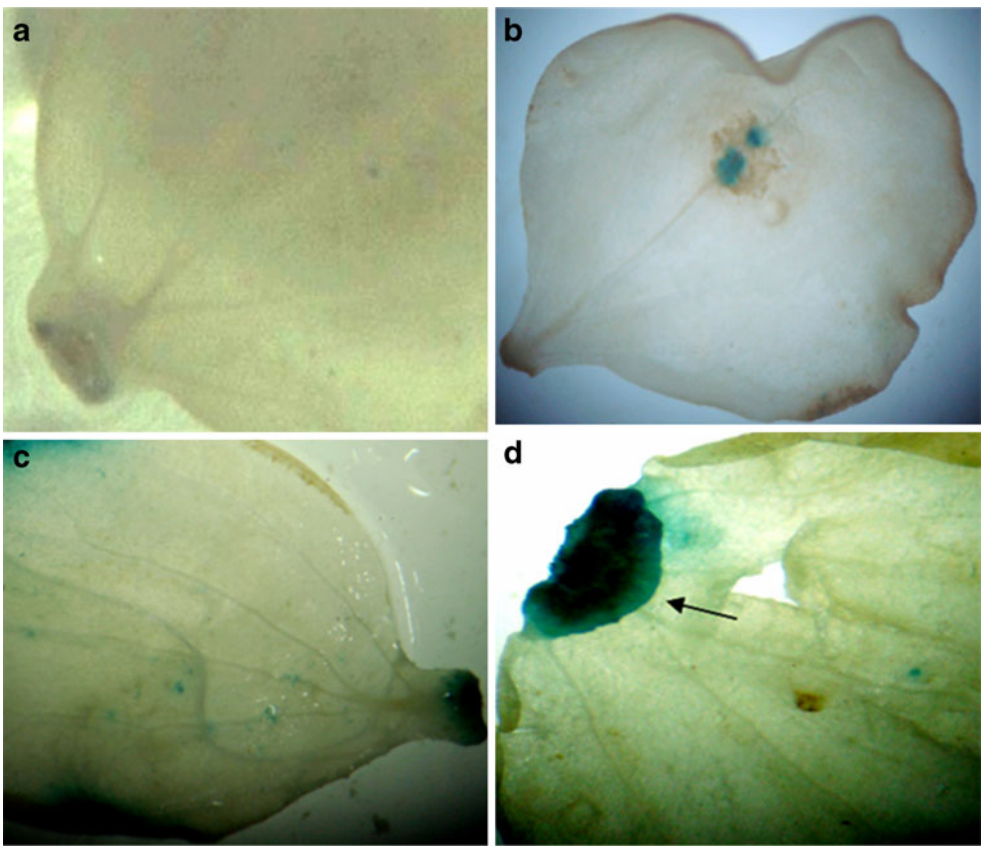

Fig. 4 β-glucuronidase (GUS) transient assay of cotyledonary petioles of jute (10×). **a** Control explant. **b** Untransformed explant showing GUS expression on the cotyledonary lamina. **c** Transformed explant showing GUS expression at the cut end of the CP. **d** GUS expression showing large blue patches at the cut surface of the CP

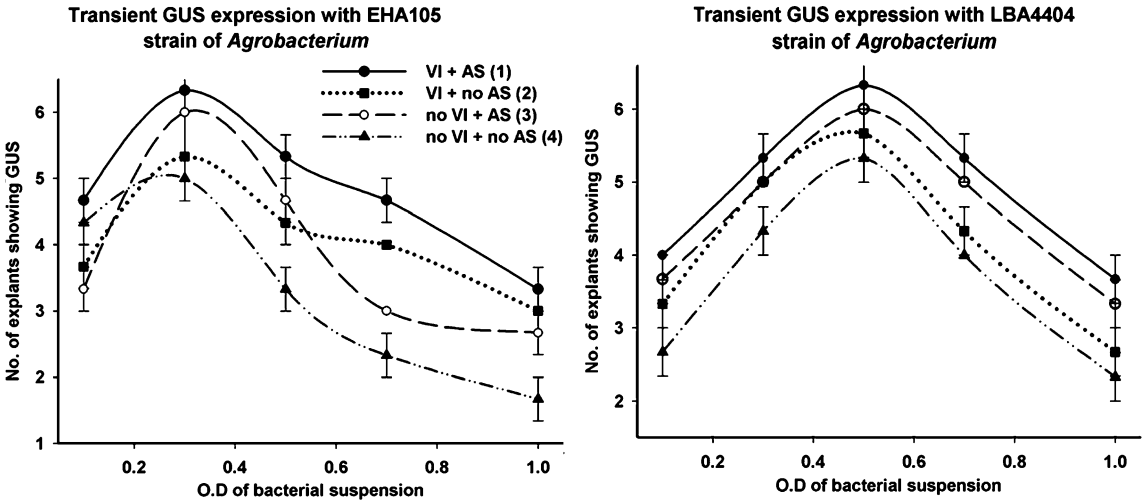

Fig. 5 Graph illustrating effect of various parameters affecting transformation efficiency by transient GUS assay

We chose to use EHA105 and LBA4404 in our experiments. Both the strains, EHA105 or LBA4404 were effective in T-DNA transfer. Bacterial concentration played a major role in efficient plant transformation. An excess of bacteria around the explant caused necrosis that would impair regeneration of the treated explants. Also, a lower bacterial cell density might not be sufficient to deliver the T-DNA. Hence, an optimal bacterial concentration must be maintained. EHA105, being more virulent than LBA4404, showed intense blue patches of GUS activity (Fig. 4) at the cut ends at a lower bacterial concentration of 0.3 as compared with LBA4404 that showed higher GUS activity at 0.5 OD. EHA105, the agropine type strain gave more number of GUS positives with larger and intense blue color than LBA4404 under the same cocultivation conditions. Herein, the extent of GUS expression is higher in EHA105 at bacterial concentration of 0.3 and decreased consequently with an increase in O.D. In contrast, LBA4404 at OD 0.5 showed high frequency of GUS expression, which decreased upon an increase in OD (Fig. 5). Sarker et al. (2008) used a bacterial concentration of 1.0 to 1.72 for transforming petiole attached cotyledons and mature embryos in the local cultivars of Bangladesh. However, we found that higher bacterial concentration resulted in bacterial overgrowth and necrosis of the explants. These results suggest that LBA4404 at O.D of 0.5 and EHA105 at O.D 0.3 showed better transformation efficiency in *C. capsularis* cv. JRC321.

Effect of vacuum infiltration on transformation

Technique of vacuum infiltration in plant transformation was used as early as 1993 for *in planta* transformation of *Arabidopsis* (Bechtold et al. 1993). Many crop plants like cotton (Haq 2004), kenaf (Srivatanakul et al. 2001) have been transformed with vacuum infiltration using the *in planta* method of transformation. Vacuum creates a negative pressure in the plant tissues and decreases the air spaces present between the cells facilitating the infiltration of bacterial cells into the intercellular spaces facilitating the relocation of bacteria along with medium into the plant tissues that are to be transformed. In our treatments, some of the explants were vacuum infiltrated for 15 min and some were subjected to suspension for the same time. We observed that prolonged exposure of the explants to vacuum lead to hyperhydricity and necrosis. However, vacuum infiltrating the petioles for 15 min showed an increase in transient transformation frequency, when compared to suspension cultures (Fig. 5) showing the beneficial effects of vacuum infiltration in jute transformation.

Transient GUS expression

The highest infection frequency of cotyledonary petioles was obtained with bacterial strains EHA105 (OD 0.3) or LBA4404 (OD 0.5) by subjecting the tissues for vacuum and supplementing with 100 μM AS. This resulted in an increase in large blue areas at the cut end of the petioles indicating enhanced gene transfer. These results establish that *A. tumifaciens* can be employed for stable genetic transformation in cotyledonary petioles of white jute, since plant regeneration from this species is successful.

Further experiments are in progress for the development of stable transgenic white jute using *Agrobacterium* based vectors.

Conclusions

Though there are several reports on shoot bud regeneration from the cotyledonary petioles of some genotypes in *C.*

capsularis, none of them dealt with the production of complete plantlets except the report of Sarker et al. (2007). The protocol discussed could not be applied to Indian jute cultivars. To the best of our knowledge, this is the first report of an efficient regeneration system using cotyledonary petioles of a local Indian variety with high frequency of regeneration. The present reported protocol is simple and rapid, and leads to the establishment of in vitro regenerated plantlets efficiently under greenhouse conditions. We have also shown that the shoot buds were regenerated de novo on the explants after culture initiation through histological analysis.

Most of the reports on white jute transformation did not provide confirmatory Southern analysis proving no integration of transgene. The binary vector pCAMBIA2301 was mobilized into *Agrobacterium* strain and is used in transient GUS expression to identify the most appropriate conditions for white jute transformation. The transient GUS assay offers an easy and reliable approach for establishing optimal conditions for transformation. The protocol describes an efficient method of transformation by optimizing parameters like bacterial strain, method of cocultivation, bacterial OD, use of acetosyringone in the suspension. Taking into account the GUS frequency from *Agrobacterium* infection, cotyledonary petiole explants appeared to be good for stable genetic transformation of Jute, JRC321.

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