

**PLANT REGENERATION AND TRANSFORMATION  
IN KENAF (*Hibiscus cannabinus*)**

By

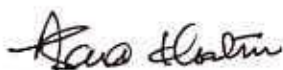
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A Thesis

Submitted to the Faculty of Agriculture,  
Sher-e-Bangla Agricultural University, Dhaka,  
in partial fulfillment of the requirements  
for the degree of

**MASTER OF SCIENCE  
IN  
GENETICS AND PLANT BREEDING  
SEMESTER: JUNE, 2008**

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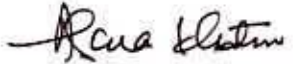
CERTIFICATE

*This is to certify that thesis entitled, "PLANT REGENERATION AND TRANSFORMATION IN KENAF (*Hibiscus cannabinus*)" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN GENETICS AND PLANT BREEDING, embodies the result of a piece of bona fide research work carried out by SHAMSUNNAHER, Registration No.: 01052 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

Dated: June, 2008

Place: Dhaka, Bangladesh

  
.....

(Dr. Asma Khatun)  
Supervisor



*Dedicated to  
My  
Beloved Parents*

## ACKNOWLEDGEMENTS

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

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

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

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

*The author takes opportunity to express her sincere thanks and profound gratitude to professor Abu Akbar Mia, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka, for his enormous help, guidance and suggestions during the research period.*

*Special appreciation and warmest gratitude are extended to Dr. Md. Sarowar Hossain, Professor of Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka who provided creative suggestions, guidance and constant inspiration from the beginning to the completion of the work. His contribution, love and affection would persist in the memory of the researcher for countless days.*

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

*The author humbly thankful to Md. Abdul Gaffar, MS student of Genetics and Plant Breeding, SAU, Dhaka, for his cordial cooperation during the research period.*

*The author feel much pleasure to convey the profound thanks to her friends, Shima, Suma, Limon and other well wishers for their cooperation, cheerfulness and help during the on going of the research. She particularly thankful to Hasem bhai and Shafiq bhai who heavily encourage her to undertake and complete this research and thesis work.*

*The author thankfully remembers the students of the Genetics and Plant Breeding for their cooperation in the entire period of study. He also feels pleasure to all staffs and workers of Genetics and Plant Breeding Department, SAU for their valuable and sincere help in carrying out the research work.*

*Eventually, the author is ever grateful and expresses her special appreciation and indebtedness to her beloved parents whose sacrifice, inspiration, encouragement and continuous blessing paved the way to her higher education. She is also grateful to her brothers, sister, grandmother, and other relatives who continuously prayed for her success and without whose love, affection inspiration and sacrifice this work would not have been completed.*

*Dated: June, 2008  
Place: SAU, Dhaka.*

*The Author*



## LIST OF ABBREVIATIONS

| Symbols           | Acronyms  |
|-------------------|---|
| %                 | Percentage  |
| °C                | Degrees Celsius   |
| 0.1 N             | 0.1 Normal  |
| ANOVA             | Analysis of variance  |
| IAA               | Indol acetic acid   |
| BAP               | 6-benzyl amino purine   |
| BBS               | Bangladesh Bureau of Statistics                                 |
| CaMV              | Cauliflower Mosaic Virus  |
| cm                | Centimeter  |
| CIP               | International Potato Centre                                     |
| CV                | Coefficient of variation  |
| DMRT              | Duncan's Multiple Range Test                                    |
| dw                | Distilled Water   |
| e.g.              | Exempli gratia (by way of example)                              |
| <i>et al.</i>     | et alu=other people   |
| etc.              | et cetera (means and the rest)                                  |
| FAO               | Food and Agriculture Organization                               |
| Fig.              | Figure  |
| g                 | Gram  |
| gl <sup>-1</sup>  | Gram per litre  |
| GDP               | Gross Domestic Product  |
| GUS               | β-glucuronidase   |
| ha                | Hectare   |
| HCl               | Hydrochloric acid   |
| HgCl <sub>2</sub> | Mercuric Chloride   |
| hrs.              | Hours   |
| i.e.              | ed est. (means That is)   |
| IARI              | Indian Agricultural Research Institute                          |
| ICRISAT           | International Crop Research Institute for the Semi-arid Tropics |

## LIST OF ABBREVIATIONS

| Symbols                       | Acronyms  |
|-------------------------------|---|
| IRRI                          | International Rice Research Institute                                     |
| j.                            | Journal   |
| LB                            | Left border   |
| mg <sup>l</sup> <sup>-1</sup> | Milligram per litre   |
| ml                            | Mili litre  |
| MS                            | Murashige and Skoog   |
| MSO                           | Hormone free Murashige and Skoog  |
| Na <sub>2</sub> -EDTA         | Sodium salt of ferric ethylene diamine tetraacetate                       |
| NAA                           | α-naphthalene acetic acid   |
| NaCl                          | Sodium chloride   |
| NaOH                          | Sodium hydroxide  |
| No.                           | Number  |
| NOS                           | Nopaline synthase   |
| nptII                         | Neomycin phosphotransferase II  |
| NS                            | Non significant   |
| pH                            | Negative logarithm of hydrogen ion concentration (-log [H <sup>+</sup> ]) |
| req.                          | Required  |
| RB                            | Right border  |
| <i>Spp.</i>                   | Species (plural)  |
| t                             | Ton   |
| T-DNA                         | Transfer DNA  |
| TK                            | Taka  |
| UK                            | United Kingdom  |
| USDA                          | United States Department of Agriculture                                   |
| UV                            | Ultra violet  |
| V                             | Volt  |
| var.                          | Variety   |
| via                           | By way of   |
| viz.                          | Namely  |
| vir                           | Virulence   |

## LIST OF ABBREVIATIONS

| Symbols | Acronyms   |
|---------|--|
| v/v     | Volume per volume                                    |
| w/v     | Weight per volume                                    |
| w/w     | Weight per weight                                    |
| X-gluc  | 5-bromo-4-chloro-3-indoyl- $\beta$ -D-glucuronidase. |
| Y       | Year(s)  |
| YMB     | Yeast Mannitol Broth                                 |
| $\mu$ g | Microgram  |





# Plant Regeneration and Transformation in Kenaf (*Hibiscus cannabinus* L.)

By

SHAMSUNNAHER

## ABSTRACT

Two different sets of experiments were conducted at the Biotechnology Laboratory, Department of Genetic Resources and Seed Division, Bangladesh Jute Research Institute, Dhaka during the period of January 2007 to November 2007. In the first experiment, a detailed investigation was carried out to study the callus induction ability and subsequent plant regeneration protocol for two varieties of kenaf (HC-2 and HC-95) using cotyledon with attached petioles as explants. In the second experiment, investigation was carried to study *Agrobacterium* mediate genetic transformation. Two different investigations were made in the first experiment to show the effect of different age of explants and BAP concentration in plant regeneration of kenaf. Five different ages (6, 8, 10, 12 and 14 days) explants and seven different BAP concentrations were used and wide ranges of variation were observed. Highest callus production (91.7%) and shoot regeneration (85.3%) were found in HC-2 when 10 days old explants were used. Both HC-2 and HC-95 showed the maximum callus initiation (91.7%) and the highest shoot regeneration (91.7%) in HC-2 when MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IAA was used. *Agrobacterium tumefaciens* strain LBA4404 was used for transformation, containing selectable marker gene nptII conferring resistance to kanamycin and GUS reporter gene also. Between the varieties, HC-2 showed the highest response to GUS expression (90.0% positive). Transgenic shoots produced roots and transferred to the soil successfully.



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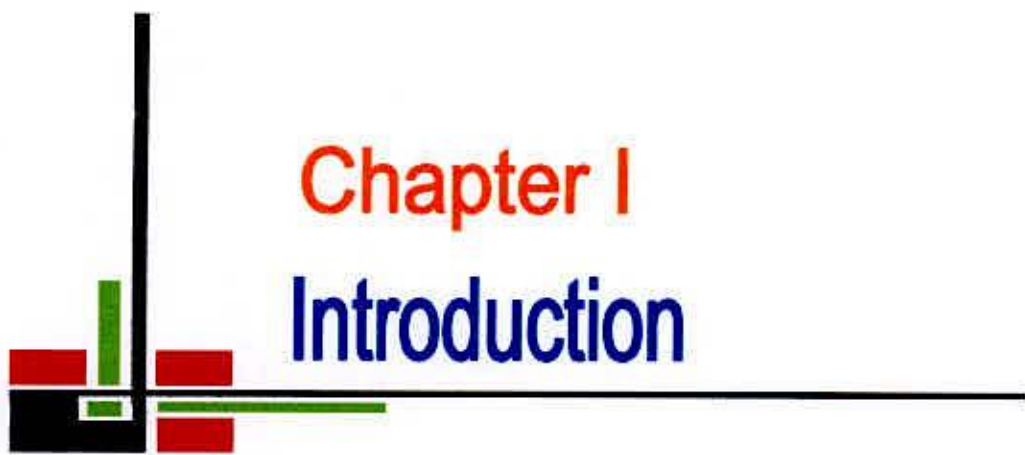




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# Chapter I

## Introduction

## CHAPTER I INTRODUCTION

---

Kenaf (*Hibiscus cannabinus* L.) is a fibre plant native to East-Central Africa where it has been grown for several thousand years for food and fibre (LeMahieu *et al.*, 2003). In the recent years, there has been an increasing trend in both area and production of kenaf in Bangladesh. However, the yield per unit area remains unsatisfactory. In Bangladesh, 963.00 thousand tons of kenaf were produced from 499.80 thousand hectares of land in 2003-2004 (FAO, 2004). The average yield of kenaf in Bangladesh is 1.92 t/ha which is much lower than that of many other kenaf growing countries of the world such as India, Indonesia, China, Australia and USA.

Kenaf is a short-day, annual herbaceous plant cultivated for the soft bast fibre in its stem (Dempsey, 1975). Kenaf grows in tropical and temperate climates and thrives with abundant solar radiation and high rainfall. Under good conditions, kenaf can grow to a height of five to six meters in six to eight months and produce up to 30 tones per hectare of dry stem material (Wood, 2003). Kenaf yields approximately three to five times as much fibre as southern pine (Le Mahieu *et al.*, 2003).

The traditional use of kenaf focuses on its fibre production, such as making ropes, sacs, canvases, and carpets (Li, 1980). However, new applications of kenaf have recently been developed such as pulping and papermaking, board making, absorbents and potting media, filtration, textiles, and livestock feed. The commercial success of kenaf has important potential economic and environmental benefits in the areas of soil remediation, toxic waste clean up, removal of oil spills on water, reduced chemical and energy use for paper production, greater recycled paper quality, reduced soil erosion due to wind and water, replacement or reduced use of fibreglass in industrial products, and the increased use of recycled plastics (Webber *et al.*, 2002a).

One of the major constraints to increase kenaf productivity is the non-availability of modern varieties, as well as, infection by fungi, bacteria, virus, nematode and many other environmental factors which may exert a deleterious effect on yield, marketable quality, germplasm conservation, distribution and international exchange (Reichert *et al.*, 1999).

To improve the important agronomic characters of kenaf, conventional breeding methods were practiced. Traditional breeding methods are employed to overcome this problem in many countries of the world including Bangladesh. However, it is time consuming and has a chance of failure. Many undesired genes also transmit. The limitations of conventional breeding include narrow genetic base of the cultivated species, the length of time needed for successfully developing crop cultivars, the difficulty in breaking gene linkages between useful and useless traits etc. In order to produce desirable lines of kenaf with good growth, high fibre yield and higher biomass production an alternate technique is necessary (Reichert *et al.*, 1994, 1996).

Biotechnology may be an alternative way to overcome this problem rapidly. For this, suitable protocol of plant regeneration and transformation is needed. Biotechnology is a recently developed novel approach, which includes a range of techniques. Together these techniques comprise a powerful technical force to produce or modify biological products according to specific objectives. Plant tissue culture, a branch of biotechnology offers an efficient method for rapid propagation, production of pathogen-free material and plant germplasm preservation. The potential rate of tissue culture in plant breeding has been widely recognised and it is generally used as an experimental tool for crop improvement of numerous economic and food crops during the last 20 years using these techniques, particularly in those for which conventional crop breeding has been less effective.

The exploitation of heritable somaclonal variants has been used in various plant improvement strategies (Larkin *et al.*, 1982; Evans 1989; Larkin *et al.*, 1989; Phillips *et al.*, 1994). Recently, plant regeneration has already been reported from the explants of kenaf, which assures the exploitation of the species in tissue culture and genetic transformation system.

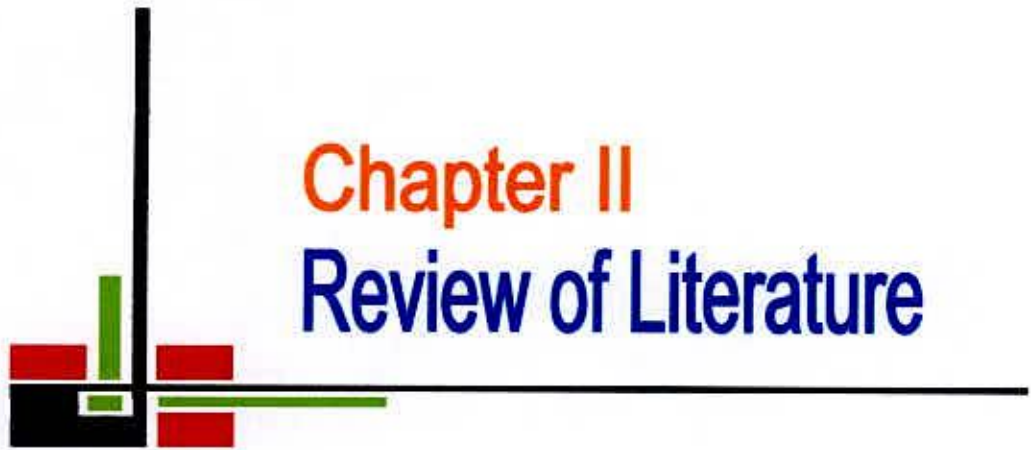
Genetic modification of plants using recombinant DNA technologies holds the promise of increased crop productivity, product quality and reduced dependence on chemical inputs for pest control (Asano *et al.*, 1991) Modern plant genetic engineering involves the transfer of desired genes into the plant genome and then regeneration of a whole plant from the transformed tissue. Currently, the most widely used method for transferring genes into plants is *Agrobacterium*-mediated transformation.

Improvement of a species through genetic engineering includes appropriate regeneration and transformation technique. High frequency regeneration of plants from *in vitro* cultured tissues and cells is a pre-requisite for successful application of tissue culture and genetic engineering technologies for crop improvement. Both, callus induction and plant regeneration from explants require the presence of appropriate combinations and concentrations of plant growth regulators in the culture media (Ehsanpour *et al.*, 2000; Fiegert *et al.*, 2000; Ahn *et al.*, 2001).

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

**The specific objectives of this research programme were:**

- To establish an efficient and repeatable plant regeneration system from the explants of kenaf.
- To establish an protocol for genetic transformation in kenaf through *Agrobacterium* strains.



## Chapter II

# Review of Literature

## CHAPTER II REVIEW OF LITERATURE

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Kenaf yields a soft fibre from the stem that is very similar to jute. Along with a closely related species called roselle (*Hibiscus sabdariffa* L.), the two species account for one-third of the world production of soft fibres used for packaging. Kenaf is rapidly replacing jute, because the crop has less intensive labour requirements, is cheaper to produce, may be grown on a wide range of soils under varied climatic conditions, and is not necessarily competitive with food crops. While kenaf is somewhat coarser than jute, it has greater tensile strength, is lighter in colour, and has greater resistance to moisture (Dempsey, 1975).

Essentially, kenaf is a traditional, third world crop that is poised to be introduced as a new, annually renewable source of industrial fibre in the so called developed economies (Taylor, 2003).

Traditional breeding methods are employed to overcome this problem in many countries of the world including Bangladesh. However it is time consuming and has a chance of failure and many undesired genes also transmit. Biotechnology may be an alternative way to overcome this problem rapidly. Recent advances in tissue culture and recombinant DNA technology have opened new avenues in transformation of higher plants, which consequently produced many transgenic plants with new genetic properties. Establishment of an efficient plant regeneration system from the explants of jute/kenaf is a prerequisite to create variability and to introduce foreign genes into this crop through genetic transformation (Khatun, 1998).

One of the most efficient methods of gene transfer for achieving goal is *Agrobacterium* mediate transformation. A brief review of works done on plant regeneration of kenaf is given bellow.

## 2.1. *In vitro* Plant Regeneration of Kenaf

### 2.1.1. Concept of Tissue Culture

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

Conventional techniques are lengthy processes and take more time for crop improvement. The techniques of plant tissue culture have been developed as a new and powerful tool for crop improvement (Carlson, 1975) and received wide attention of modern scientists (Skirvin, 1978, Larkin *et al.*, 1982). The rapid cloning of new varieties and establishment of virus-free lines has been commercially exploited by tissue culture companies through out the world.

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

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





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

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

### **2.1.2. Tissue Culture of Kenaf (*Hibiscus cannabinus*. L)**

*In vitro* regeneration has been quite difficult among the species of kenaf and jute through tissue culture technique. It appears that kenaf and jute has a notorious recalcitrant tissue and regeneration is sporadic. Regeneration has only been reported from meristematic tissue other than totally differentiated tissue, like callus. In kenaf regeneration observed from cotyledons with attached petioles and hypocotyls where meristematic tissue present (Khatun *et al.*, 1998, 2003; Hoque, 2005).

An attempt was taken to develop a protocol of transformation of kenaf by using *Agrobacterium* strain LBA4404 (Hoque, 2005).

*In vitro* regenerated plants in some cases give rise to spontaneous mutagen induced genetic variation. These somaclonal variants in some cases can be useful for the selection of desirable genetic variants. As part of the kenaf improvement program at Mississippi State University, tissue culture is being employed as a means for introduction of new or altered traits into kenaf. Regenerants often display altered phenotypes, termed somaclonal variation (Larkin *et al.*, 1982).

Reichert and Liu, (1994) has optimized adventitious shoot regeneration protocols for kenaf, starting with internodal stem and leaf sections. They regenerated three new varieties: Everglades 41 (E<41), Guatemala 45 (G 45) and G 48.

### 2.1.3. Callus Induction from Kenaf Varieties

A callus is an amorphous mass of loosely arranged thin-walled parenchymatous cells arising from the proliferating cells of parent tissue. Different explants and different combinations of growth regulators have an influence on callus induction from various *Hibiscus cannabinus* varieties. Various explants like cotyledons with attached petiole, hypocotyle containing meristemetic zone and shoot apices were used for regeneration of kenaf.

McLean *et al.*, (1992) a research group at Mississippi State University was first to regenerate kenaf plants *in vitro*. They were used internodal stem explants of tainung 1 on media containing different combinations and concentrations auxins and cytokinins (PGR's). Within 5 days, callus formed around the periphery of the explants. Within 30 days, adventitious shoots were developed from the callus. The shoots were excised and placed on a different medium for root formation.

### 2.1.4. Shoot Regeneration from Kenaf Explants

For shoot regeneration from kenaf cotyledons with attached petioles, BAP concentration 3.0 mg/l and IAA concentration 0.5mg/l is the best (Hoque, 2005).

A direct and simple regeneration procedure using shoot apex is reported by Zapata *et al.*, (1999). Regeneration from internodal section is reported by Liu (1994). They were taken internodal stem sections from field-grown plants of 'Everglades 41' ('E41'), surface-disinfected, then placed on a medium containing Murashige and Skoog (1962) salts, supplemented with 0.1 mg/l 1-naphthalene acetic acid (NAA) and 3.0 mg/l thidiazuron (TDZ) with pH 5.8 for shoot initiation.

Kenaf (*Hibiscus cannabinus* L.) belongs to the Malvaceae family, are used as sources of fibers. Shoot apices were taken from kenaf seedlings germinated from 3 different cultivars (Zapata *et al.*, 1999). Kenaf shoot apex size was between 2-3 mm containing the meristem, unexpanded leaves, and a small portion of the cotyledon. Shoot apices were placed on 18 different media containing full and 1/2 strength Murashige and Skoog (1962) plus vitamins, and combinations of 0, 0.1, and 1 mg/l of naphthalene acetic acid and 6-

benzyladenine (BA). The shoot apices of kenaf multiplied successfully without intervening callus formation, and no significant differences among cultivars were found. An average of 92% of the kenaf shoot apices initiated shoots and rooted in full strength Murashige and Skoog (1962) in full strength plus vitamins and 0.1 mg/l BA in 3 weeks.

An efficient protocol for plant regeneration from the cotyledons of kenaf was reported by Khatun *et al.* (2003). They obtained regenerated plants from the cotyledonary petioles of kenaf (*Hibiscus cannabinus* L. var. HC-2) on MS medium supplemented by different levels of IAA or NAA and BAP. Influence of Pluronic F-68 was studied on shoot regeneration of kenaf cotyledons with attached petioles *in vitro*. The effect was most marked in 0.1% and 0.5% level of Pluronic F-68 in addition to usual plant regeneration medium and results were found much higher than the control. Plants regenerated from the pluronic-treated cotyledons were found morphologically normal and pluronic F-68 was found to be growth stimulating agent for increasing the shoot regeneration efficiency of explants.

Purwati and Sudarmnadj (1998) studied the response of five kenaf accessions for shoot regeneration and established regeneration protocol for kenaf. From cotyledons with attached plumules. Purwati and Sudannadji (1999) used MS based medium containing BAP (2 mg/l) and GA3 (0.5 mg/l) for callus induction. Calli produced in this culture were then transferred into MS-based medium containing BAP (2 mg/l) and GA3 (5 mg/l) for shoot initiation.

*In vitro* regeneration method is also developed for kenaf by Herath *et al.* (2004). Multiple shoots are induced from shoot tips and cotyledonary nodes of kenaf cultured on MS medium treated with benzyl adenine (BA). The number of shoots regenerated varied with the cultivar, explant, and the BA concentration. Highest number of shoots (11/explant) was developed in cv tainung 2 (T2) shoot tips cultured in MS medium supplemented with 2 mg/l BA (Herath *et al.* 2004). Srivatanakul *et al.* (2000) developed a medium that stimulates multiple shoot initiation from explants of kenaf (*Hibiscus cannabinus* L). Adventitious shoot formation on a shoot induction media supplemented with combinations of 2, 4-D

(0, 0.5, 2.3  $\mu\text{mol/l}$ ) and thidiazuron (0, 1, 5, 20  $\mu\text{mol/l}$ ) was evaluated. Multiple shoot induction medium with 1  $\mu\text{mol}$  TDZ/l resulted the highest number of regenerated shoot per explant.

### **2.1.5. Root Induction**

Herath *et al.*, (2004) studied the nature of regenerated shoot elongation and shoot induction character. Shoot elongation and rooting is obtained simultaneously in half strength MS basal medium without plant growth regulators. About 98% of the rooted plants are grown to maturity under green house conditions.

## **2.2. *Agrobacterium* - Mediated Genetic Transformation in Kenaf**

### **2.2.1. Concept of Genetic Transformation**

Techniques of tissue culture can play an important role in overall improvement of crop, but contributed a little in the production of disease and pest resistant plants. To overcome such problems in crop improvement genetic transformation of crop plants has been evolved, which offers the ability to introduce single new character into a plant cultivar without altering of its existing traits (Gardner, 1993). Thus, genetic transformation provides an exciting new technology to supplement traditional crop improvement programmes and together these approaches should accelerate the development of a new plant variety, which is not possible through breeding and tissue culture alone.

In all genetic transformation experiments, specific reporter gene and one or more selectable marker gene are required to be incorporated into the plant cells prior to the integration of gene/genes of interest. In this case, GUS-A ( $\beta$ -glucuronidase) gene and neomycin phosphotransferase II termed as npt II (kanamycin resistant), gene have been used as reporter and selectable marker genes, respectively. These reporter genes can be recognized in plant tissue with the help of selectable agents, confirming transformation of the plant tissue (through histochemical GUS assay). So, in this way, one can understand that the plant tissues subjected for transformation have really been transformed or not (Gardner, 1993).

### 2.2.2. *Agrobacterium tumefaciens* - as a Vector

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



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



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

The T-DNA carries genes for the biosynthetic enzymes for the production of unusual amino acids, typically octopine or nopaline. It also carries genes for the biosynthesis of the plant hormones, auxin and cytokinins. By altering the hormone balance in the plant cell, the division of those cells cannot be controlled by the plant, and tumors form (Pan *et al.*, 1993). The ratio of auxin to cytokinin produced by the tumor genes determines the morphology of the tumor (root-like, disorganized or shoot-like).

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

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

The genes to be introduced into the plant are cloned into a plant transformation vector that contains the T-DNA region of the disarmed plasmid, together with a selectable marker (such as antibiotic resistance) to enable selection for plants that have been successfully transformed. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die (Das and Xie, 1998).

*Agrobacterium* strains contain a large mega plasmid (more than 200 kb). Which plays a key role in tumor induction and for this reason it was named Ti plasmid or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the T-DNA; a mobile segment of Ti and Ri plasmid is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a cis element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and region for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regular organized in six operons that are essential for the T-DNA transfer (*vira*, *vir B*, *VirD* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoot, 1992; Zupan and Zambryski 1995, Jeon *et al.*, 1998).

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

### **2.2.3. *Agrobacterium tumefaciens* T- DNA Transfer Process**

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



Although the gene transfer mechanisms remain largely unknown, great progress has been obtained in practical implementation of transformation protocols for both dicotyledonous and monocotyledonous plants. Particularly important is the extension of this single-cell transformation methodology to monocotyledonous plants. This advance has biological and practical implications. Firstly, because of advances of *A. tumefaciens*-mediated gene transfer over the direct transformation methods, which were the only way for genetic manipulation of economically important crops as cereals and legumes. Second, it has been demonstrated that T-DNA is transferred to dicot and monocot plants by an identical molecular mechanism. This confirmation implies that any plant can potentially be transformed by this method if suitable transformation protocol is established (Wood *et al.*, 2001).

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

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

T-DNA transfer process to plant cells has three important findings for the practical use of the process in plants transformation.

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



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

#### **2.2.4. Plant transformation mediated by *Agrobacterium tumefaciens***

Kenaf is an environmentally friendly crop; however, commercial production of kenaf is hindered by weed competition at the seedling stage. Herbicide resistant kenaf cultivars would reduce seedling weed competition and make growing kenaf more profitable (Ehsanpour *et al.*, 2000).

Traditional plant improvement relies on the use of sexually-compatible germplasm for introduction of new/altered traits via cross-pollinations. However, this type of improvement is limited to traits already present in the compatible germplasm. Development of genetic engineering protocols will allow the introduction of traits from any living organism into kenaf (Zapata *et al.*, 1999).

*Agrobacterium*-mediated transformation is the most widely used method to transfer genes into plants. Transformation is typically done on a small excised portion of a plant known as an explant. The small piece of transformed plant tissue is then regenerated into a mature plant through tissue culture techniques (Fraleley *et al.*, 1983). Callus and cell suspension cultures were initiated and maintained in sugarcane alone, cvNIF4 (*Saccharum. spp.* hybrid) and were used as starting material (Matsuoka *et al.*, 2000).

The first plant transformed by *Agrobacterium tumefaciens* was tobacco (Herrera-Estrella, 1983). Since that crucial moment in the development of plant science, a great progress in understanding the *Agrobacterium*-mediated gene transfer to plant cells has been achieved. However, *Agrobacterium tumefaciens* naturally infects only dicotyledonous plants and many economically important plants,

including the cereals, remained accessible for genetic manipulation during long time. For these cases alternative direct transformation methods have been developed (Lörz *et al.*, 1985; Debnath *et al.*, 1996; Arencibia 1995).

However *Agrobacterium*-mediated transformation has remarkable advantages over direct transformation methods in reducing the copy number of the transgene, potentially leading to fewer problems with transgene cosuppression and instability (Koncz *et al.*, 1994, Hansen *et al.*, 1997). In addition, It is a single-cell transformation system and avoids the obtainment of mosaic plants, which are more frequent when direct transformation is used (Enríquez-Obregón *et al* 1997, 1998).

Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the most major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants (Birch, 1997), using *Agrobacterium*-mediated or direct transformation methods. The idea, that some species can not accept the integration of foreign DNA in its genome and lack the capacity to be transformed is unacceptable under the increasing number species that have been transformed.

The optimisation of *Agrobacterium tumefaciens*-plant interaction is probably the most important aspect to be considered. It includes the integrity of bacterial strain its correct manipulation as warranty of the virulence machine integrity and the study of reaction in wounded plant tissue, which may develop necrotic process in the wounded tissue or affect the interaction and release compounds inducers or repressors of *Agrobacterium* virulence system. The type of explant is also important fact and it must be suitable for regeneration allowing the recovering of whole transgenic plants. The establishment of method for efficient regeneration for one particular species is crucial for its transformation.

Srivatanakul *et al.*, (2001) reported that several factors are important in establishing a transformation system for kenaf. They investigate the influence of *Agrobacterium* strain, temperature, host tissue wounding, acetosyringone. VirG/virE genes and host cell division on T-DNA expression in the kenaf shoot

apex. Three *Agrobacterium* strains were tested and *A. tumefaciens* LBA4404 significantly ( $\alpha=0.05$ ) yielded a high number of shoots surviving on selection medium: no shoots survived with EHA101S or Z707S. There was no significant difference ( $\alpha=0.05$ ) in transient T-DNA expression between 28 °C and 25 °C; however, shoots did not survive in 16 °C or in 19 °C co-cultivation temperatures. Shoot apex survival was increased significantly ( $\alpha=0.05$ ) when virulence genes and a cytokinin, TDZ, were combined. Optimal conditions for shoot apex T-DNA transfer and expression were co-cultivation with LBA 4404 containing virG/virE at room temperature, and 200 mol/L acetosyringone.

As a first step in the development of a successful *Agrobacterium tumefaciens* mediated transformation method for kenaf, factors influencing the successful T-DNA integration and expression (as measured by the GUS expression) were investigated by Herath *et al.*, (2005). Transformation was carried out using two kenaf cultivars and *Agrobacterium* strain EHA 105 carrying different vectors, plasmid pIG 121-Hm or pEC:gus. Pre-culturing the explants for 2 days in benzyl adenine containing medium, and wounding the explant before inoculation were found to enhance the transient GUS expression. Previous research by others (Banks *et al.*, 1993) proved that kenaf could be genetically engineered.

The mentioned aspects are important to establish transformation procedure for any plant but particularly for those species categorised as recalcitrant. In this category have been included cereals, legumes and woody plants, which are very difficult to transform or remain untransformed. Many species originally considered in this category has been transformed in recent years. One of these species, sugarcane, has been transformed (Arencibia *et al.*, 1998).

A number of tropical and sub-tropical crops are now amenable to gene transfer system by genetic engineering using *Agrobacterium*-mediated gene delivery system. A list has been compiled by Gardner (1993): Potato (Block, 1988; Cardi *et al.*, 1992; Shahin and Simpson, 1986), citrus (Hidaka *et al.*, 1990), potato maize (Gould *et al.* 1991). Lettuce (Debnath *et al.*, 1996), jute (Hossain *et al.*, 1995), Kiwifruit (Uematsu *et al.*, 1991, Rugini *et al.*, 1991; Janssen, 1991), strawberry (Nehra *et al.*, 1990), tobacco (An *et al.*, 1985), tomato (Fillali *et al.*, 1987); Chilli (Hyde *et al.*, 1996).



## Chapter III

# Materials and Methods

## CHAPTER II MATERIALS AND METHODS

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The present investigation was carried out in the Biotechnology Laboratory of the Department of Cytogenetics, Genetic Resource and Seed Division, Bangladesh Jute Research Institute from January 2007 to November 2007. A part of the current study was conducted at Biotechnology laboratory, Bangladesh Sugarcane Research and Training Institute. Two different experiments were conducted to fulfill the objectives of the present study.

**Experiment 1:** *In vitro* regeneration of two varieties of kenaf

(*Hibiscus cannabinus*)

**Experiment 2:** *Agrobacterium*- mediated genetic transformation of two kenaf varieties

### 3.1. Experimental Materials

Cotyledons with attached petioles obtained from two varieties of kenaf (HC-2 and HC-95) and *Agrobacterium* strain LBA4404 were used for kenaf regeneration and transformation experiment.

### 3. 2. Sources of Experimental Materials

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

### 3. 3. Location, Time and Duration

To achieve the objectives, the experiments were conducted in the Biotechnology Laboratory, Department of Cytogenetics, Bangladesh Jute Research Institute (BJRI), Dhaka, during the period from January 2007 to November 2007.

### **3.4. *Agrobacterium* Strain and Plasmid**

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used for infecting explants in the transformation experiment. The strain contains plasmid pB1121 of 14 kDa (binary vector). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct:

- The *uidA* gene, the reporter gene, encoding GUS ( $\beta$ -glucuronidase), driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation.
- The *npt II* gene encoding neomycin phosphotransferase II (npt II) conferring *kanamycin* resistance, driven by NOS promoter and NOS terminator.
- The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid (132 kDa) containing the *virulence* genes.

### **3. 5. Media Used**

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

#### **3. 5.1. Seed Germination**

For the germination of seeds on both agar based MS media and clinical cotton based MS media were used.

#### **3. 5. 2. Callus Induction and Regeneration**

For the induction and maintenance of callus, MS (Murashige and Skoog, 1962) medium containing macronutrients, micronutrients and vitamins with different concentrations and combinations of IAA and BAP were used.

### **3.5.3. *Agrobacterium* Culture and Inoculation**

YMB (Yeast extract Mannitol Broth) medium was used with kanamycin as antibiotic to grow genetically engineered *Agrobacterium tumefaciens* strain. Agar solidified YMB medium was used for the maintenance of the *Agrobacterium* and liquid YMB medium was used for infection.

### **3.5.4. Co-cultivation**

MS medium with growth regulators and cefotaxime were used for co-cultivation

### **3.5.5. Post-cultivation and Callus Induction**

MS medium supplemented with 0.5 mg/l IAA, 3.0 mg/l BAP and 500 µg/ml cefotaxime was used for this purpose.

### **3.5.6. Selection and Regeneration**

MS medium supplemented with 0.5 mg/l IAA, 3.0 mg/l BAP, 50 µg/ml kanamycin

## **3.6. Methods of Media Preparation**

### **3.6.1. Preparation of Stock Solutions**

Separate stock solutions for macronutrients, micronutrients, iron, vitamins, growth regulatory etc. were prepared and stored appropriately for use. Chemical composition of macro and micronutrients, iron, vitamins and growth regulators is given below (Table 1).

**Table 1. Constituents of stock solution for MS (Musashige and Skoog, 1962) medium**

| <b>Constituents</b>                                |                            |                              |
|--|----------------------------|------------------------------|
| <b>a) Macronutrients</b>                           | <b>Concentration(mg/l)</b> | <b>Concentration(g/l)10X</b> |
| KNO <sub>3</sub>                                   | 1900.00                    | 19.00                        |
| NH <sub>4</sub> NO <sub>3</sub>                    | 1650.00                    | 16.50                        |
| KH <sub>2</sub> PO <sub>4</sub>                    | 170.00                     | 1.7                          |
| CaCl <sub>2</sub> .2H <sub>2</sub> O               | 440.00                     | 4.4                          |
| MgSO <sub>4</sub> .7H <sub>2</sub> O               | 370.00                     | 3.7                          |
| Na <sub>2</sub> EDTA                               | 37.30                      | 0.373                        |
| <b>b) Micronutrients</b>                           |                            | <b>100X</b>                  |
| MnSO <sub>4</sub> .4H <sub>2</sub> O               | 22.30                      | 2.23                         |
| H <sub>3</sub> BO <sub>3</sub>                     | 6.20                       | 0.62                         |
| ZnSO <sub>4</sub> .7H <sub>2</sub> O               | 8.60                       | 0.86                         |
| KI   | 0.83                       | 0.083                        |
| Na <sub>2</sub> Mo <sub>4</sub> .2H <sub>2</sub> O | 0.25                       | 0.025                        |
| CuSO <sub>4</sub> .5H <sub>2</sub> O               | 0.025                      | 0.0025                       |
| CoCl <sub>2</sub> .6H <sub>2</sub> O               | 0.025                      | 0.0025                       |
| FeSO <sub>4</sub> .7H <sub>2</sub> O               | 56.00                      | 0.56 (g/l)                   |
| <b>c) Organic sources</b>                          |                            | <b>(mg/l)100X</b>            |
| Glycine  | 2.00                       | 200                          |
| Nicotinic acid                                     | 0.50                       | 50                           |
| Pyridoxin-HCl                                      | 0.50                       | 50                           |
| Thimine-HCl  | 0.10                       | 10                           |
| Myo-inositol                                       | 100.00                     | 0.1                          |
| <b>d) Agar</b>                                     | 800.00                     | 8.00                         |
| <b>e) Sugar</b>                                    | 30000.00                   | 30.00                        |



Stock solution for growth regulators were prepared separately by dissolving the desired quantity of ingredients in appropriate solvent. The required final volume was made with distilled water for ready to use to expedite the preparation of the medium wherever needed.

### **Stock Solution I (Macronutrients)**

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

### **Stock Solution II (Micronutrients)**

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

### **Iron Source**

The required amount  $\text{FeSO}_4$  was weighted and added directly during the preparation of a medium.

### **Stock Solution III (Vitamins)**

- Each of the desired ingredients except myo-inositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water.
- Then the volume was made up to 1000 ml by further addition of distilled water.
- The solution was dispensed into 10 ml aliquots and stored at -20°C.
- Myo-inositol was used directly at the time of media preparation.

## **Stock Solutions of Hormone**

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

Auxin: Indoleacetic acid (IAA)

Cytokinin: 6-benzylamino purine (BAP)

For the preparation of stock solution or any of these hormones,

- 10 mg of each of the hormone powder was taken in a clean watch glass and dissolved in 1.0 ml of the particular solvent
- IAA was dissolved in absolute alcohol and
- BAP was dissolved in 0.1N NaOH.
- The mixture was then taken in a 100 ml volumetric flask and volume was made up to 100ml by the further addition of distilled water.
- The solution was then stored at 4°C.

### **3.6.2. Preparation of Culture Media**

Steps followed for the preparation of culture media were:

#### **3.6.2.1. Preparation of MS Media**

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

- 100 ml of macronutrients (stock solution I), 10 ml of micronutrients (stock solution II) were taken into a two litre Erlenmeyer flask on a magnetic stirrer.
- 0.056 g of Iron was added directly.
- 10 ml of vitamins stock solution were taken also.
- 450 ml distilled water was added in the flask to dissolve all the ingredients.
- 100 mg of myo-inositol was added directly to the solution and dissolved well.
- 30g of sucrose was added to this solution and agitated gently to dissolve completely.

- Different concentrations of hormone supplements were added to the solution either in single or in combinations as required and mixed well.
- MSO medium was prepared without hormone,
- pH of the medium was adjusted to 5.8 with a digital pH meter by adding NaOH or HCl (1% solution) whichever was necessary.
- The whole mixture was then made up to 500 ml with further addition of distilled water.
- Agar was dissolved in 500 ml distilled water and hot liquid agar was added with 500 ml medium.
- Required volume of hot medium was dispensed into culture vessels or conical flasks.
- After dispensing the medium the flasks were plugged with non-absorbent cotton plug and marked with different codes with the help of a permanent marker to indicate specific hormone combinations.

### **3.6.2.2. Preparation of *Agrobacterium* Culture Medium**

YMB media was used for the maintenance of *Agrobacterium* strain LBA 4404

#### **Preparation of YMB (Yeast Extract Mannitol Broth) Medium**

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

**Table 2. Constituents of YMB medium**

| <b>Ingrdients</b>                    | <b>Amount (%)</b> | <b>g/100 ml</b> |
|--------------------------------------|-------------------|-----------------|
| Mannitols                            | 1                 | 1.0             |
| East extract                         | 0.02              | 0.04            |
| MgSO <sub>4</sub> .7H <sub>2</sub> O | 0.01              | 0.02            |
| NaCl                                 | 0.05              | 0.01            |
| KH <sub>2</sub> PO <sub>4</sub>      | 0.05              | 0.05            |

Ingredients given above were taken at given composition then the pH was adjusted to 7.0 before adding agar at 1.5%.

After autoclaving, the medium was cooled to 50-55°C and antibiotic kanamycin was added at a rate of 50 mg/l and separated in Petri dishes. When the medium became solid, the dishes were prepared for bacterial culture.

For preparing bacterial suspension culture the above YMB medium without agar (i.e, liquid medium) was used.

### **3.7. Sterilization Techniques**

#### **3.7.1. Sterilization of Culture Media**

- All prepared media were autoclaved at 15 psi pressure and 121<sup>0</sup>C temperature for 20 minutes.
- For bacteria culture, YMB medium was autoclaved and poured into sterile Petri dish and sterile culture vessels (conical flask) in a laminar air flow cabinet and were allowed to cool before use.



### 3.7.2. Sterilization of Glasswares and Instruments

- Beakers, test tubes, conical flasks, pipettes, small instruments like forceps, scalpels, inoculation loops, micropipette tips, and eppendorf tubes were prepared by wrapping with brown papers before autoclave.
- Supplied water in 500 ml conical flask were plugged with cotton and wrapped with brown paper.
- Then sterilized in an autoclave at a temperature of 120 °C for 20 minutes at 15 psi pressure.

### 3.7.3. Sterilization of Culture Room and Transfer area

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

### 3.7.4. Precautions to Ensure Aseptic Condition

- All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet.
- The cabinet was switched on for at least half an hour before use and cleaned with 95% ethyl alcohol to overcome the surface contaminants.
- During the entire period of inoculation the autoclaved scalpels, forceps and inoculation loops were kept immersed into 95% ethyl alcohol contained in a test tube inside the cabinet.
- At the time of inoculation these were again sterilized by flaming them inside the cabinet.
- Both of the hands were rinsed with 95% ethyl alcohol.
- All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants.

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## **3.8. Culture Techniques**

### **3.8.1. Experiment 1**

The following culture techniques were employed in the present investigation:

1. *In vitro* seedling development
2. Explant culture
3. Subculture

#### **3.8.1.1. *In vitro* Seedling Development**

Healthy seedling production was found to be one of the major criteria for plant regeneration from kenaf explants and thereby to be successful in genetic transformation. The following steps were done for *in vitro* seedling development:

- Seeds of *H. cannabinus* (HC-2 and HC-95) were surface sterilized by immersing in absolute alcohol for 3 min. Then immersed in 0.1% Mercuric Chloride ( $\text{HgCl}_2$ ) for 20 min.
- Seeds were thoroughly washed with autoclaved water for 5-6 times.
- The sterilized seeds were transferred in a 500 ml conical flasks containing both 50 ml of hormone free MS (Murashige and Skoog, 1962) agar (Sigma, UK, 0.8%, w/v) solidified medium and cotton supported liquid medium contained in a 500 ml capacity of conical flasks.
- Each flask contained 12-15 seeds and was placed in growth room with  $28 \pm 2^\circ\text{C}$  under 1500 lux fluorescent illumination with 12 hrs. alternate dark and light condition.

#### **3.8.1.2. Explants Culture**

- Cotyledons with attached petioles of kenaf were used as explant.
- Kenaf Seedlings were used for cotyledon culture after the emergence of the shoots between the cotyledons.
- It was made sure that the emerging shoots were not remained attached with the petioles.
- Four cotyledons with attached petioles were separately arranged horizontally in 250 ml conical flasks and gently pressed on the surface of the sterilized culture medium (MS medium) so that the cut end of the petioles were inserted into the medium to a depth of 2 mm.

- Various combinations and concentrations of growth regulators BAP (0, 1, 2, 3, 4, 5 and 6 mg/l) and IAA (0.5mg/l) were used.
- The cultures were maintained in a growth room with  $28\pm 2^{\circ}\text{C}$  temperature under 1500 lux fluorescent illumination of 12 hrs photoperiod.
- The conical flasks were checked daily to note the response and the development of contamination.
- Number of shoots regenerated from the cotyledons of kenaf in the culture medium was recorded after 35 days of culture.

### **3.8.1.3. Subculture**

Cultures were transferred to fresh media regularly at an interval of five to six weeks and were routinely examined for different morphogenic development. Hormone free MS medium was used as subculturing media.

### **3.8.1.4. Root Initiation**

For the root initiation explants were transferred/ subcultured to hormone free MS medium or media with out BAP.

### **3.8.1.5. Transfer to Soil**

After root production, regenerated shoots were transferred to soil.

## **3.8.2. Experiment-2**

The following techniques were used in the present investigation:

1. Selection of *Agrobacterium* strain
2. *Agrobacterium* culture
3. Explants preparation
4. Inoculation and incubation
5. Co-cultivation
6. Transfer of the selected materials to regeneration medium
7. GUS ( $\beta$ -glucuronidase) histochemical assay

### 3.8.2.1. Selection of *Agrobacterium* Strain

The bacterial strain (with the desired plasmid) used for infection and transformation study are mentioned below with their relevant characteristics.

- *Agrobacterium tumefaciens* LBA 4404 were used for current study. The Ti plasmid in strain LBA 4404 contains plasmid of 14 Kda (binary vector) pBI121.
- Binary vector contains following genes within the right border (RB) and the left border (LB) region of the construct:
- The  $\beta$ -glucuronidase gene driven by CaMV promoter and NOS (nopaline synthase) terminator.
- The neomycin phosphotransferase (npt II) derived by NOS promoter and terminator conferring kanamycin resistance for bacterial selection.
- The bacteria also contain plasmid pAL 4404 which is disarmed Ti plasmid (132 Kda) containing the virulent genes acting in trans.

An 800-bp fragment containing the 35S promoter from the Cauliflower mosaic virus (CaMV) was cloned into pBI121 to create this binary vector. Map of the binary vector is given (Figure 2 & Figure 3).



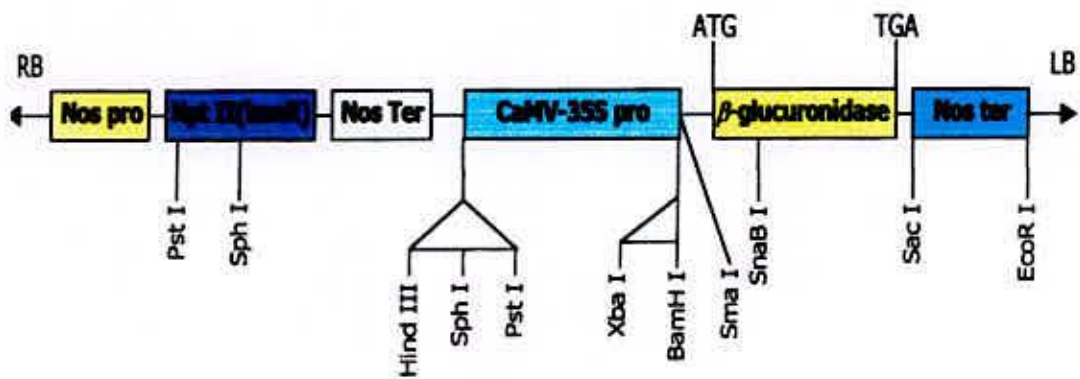
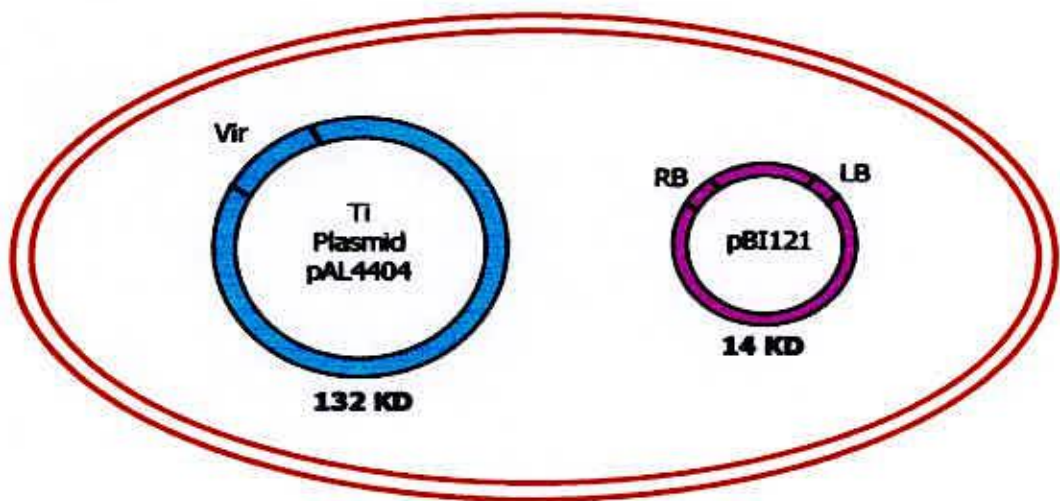


Figure 2. pBI121 – Region between left border (LB) and right border (RB)



**Figure 3. *Agrobacterium tumefaciens* LBA 4404**



### 3.8.2.2. *Agrobacterium* Culture

Two forms of culture medium (YMB medium) were used for working with the *Agrobacterium* strain. For maintaining of strain, solid YMB medium was required and liquid YMB medium were used for the infection of the explants.

#### Steps of maintenance/Streak culture of *Agrobacterium tumefaciens* strain LBA4404

- One single colony from previously maintained *Agrobacterium* stocks was streaked onto freshly prepared petri dishes containing YMB medium having kanamycin.
- Single colony was taken by sterilized loop and streaking was performed by zigzag motion on petrid dishes and sealed with parafilm.
- Petrid dishes were kept in the growth room at 28°C temperature for 24 hrs for growth and single colony formation.
- It was kept at 4<sup>0</sup>C in refrigerator for a month to check over growth. Such culture of *Agrobacterium* strain was thus ready to be used for liquid culture.
- To maintain a strain of bacterium, subculturing/streaking must be repeated at an interval of one month.

### 3.8.2.3. Explant Preparation

- Cotyledons with attached petiole obtained from *in vitro* raised seedling were used as experimental materials. Seedlings were raised following the method described in section
- Prior to transfer of all explants to co-cultivation medium explants were blotted dry with sterile tissue papers for a short period of time to remove excess of bacterial suspension.
- All the explants were maintained in co-cultivation media for 24 hours.

### 3.8.2.4. Inoculation and Incubation

The *Agrobacterium* strain grown in liquid YMB medium was used for infection and incubation.

#### **Steps For infection/Suspension culture of *Agrobacterium tumefaciens* strain LBA4404**

- Three screw capped conical flasks were taken and added each of conical flasks 50 ml of liquid YMB medium with 50 µg/ml kanamycin.
- Fresh streak cultured (not more than 7 days old) *Agrobacterium* strain LBA4404 was taken and three single colonies were selected.
- A single colony was added by sterilized inoculation loop to each conical flask from this *Agrobacterium* stock
- The flasks were than placed on a orbital shaker for inhibiting bacterial colonization.
- The conical flasks were sealed with aluminium foil and rubber band.
- The culture was allowed to grow at 28°C at 150 rpm for 24 hours to get optimum population of *Agrobacterium* for infection and co-cultivation with explants.
- Prior to this optical density of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer.
- Following the determination of density, explants were dipped into bacterial suspension ( $OD_{600} = 0.6$ ) for 1 minute before transferring them to co-cultivation medium.

## Flow diagram of Long term preservation of *Agrobacterium* in liquid culture

YMB liquid medium + kamycin + single colony from streak Plate



Shaking at 100 rpm in incubation shaker 28°C for one day



After growth of bacteria add 30% glycerol in water /  
Add equal volume of bacterial culture



Mix well and disperse in 1ml eppendorf tubes and store in freezer at -80°C

### 3.8.2.5. Co-cultivation

Following infection and incubation, the explants were co-cultured in co-cultivation medium:

- Prior to transfer of all explants to co-cultivation medium explants were blotted dry with sterile tissue papers for a short period of time to remove excess of bacterial suspension.
- All the explants were maintained in co-cultivation media for 24 hours.
- Co-cultured conical flasks containing explants were placed under fluorescent illumination for 12 hours alternate light and dark condition at  $28 \pm 2^{\circ}\text{C}$ .
- The intensity of light was maintained at 1500 lux.
- The culture vessels were checked to note the behaviour of the explants.

### 3.8.2.6. Transfer to the Nutrient Medium / Subculturing

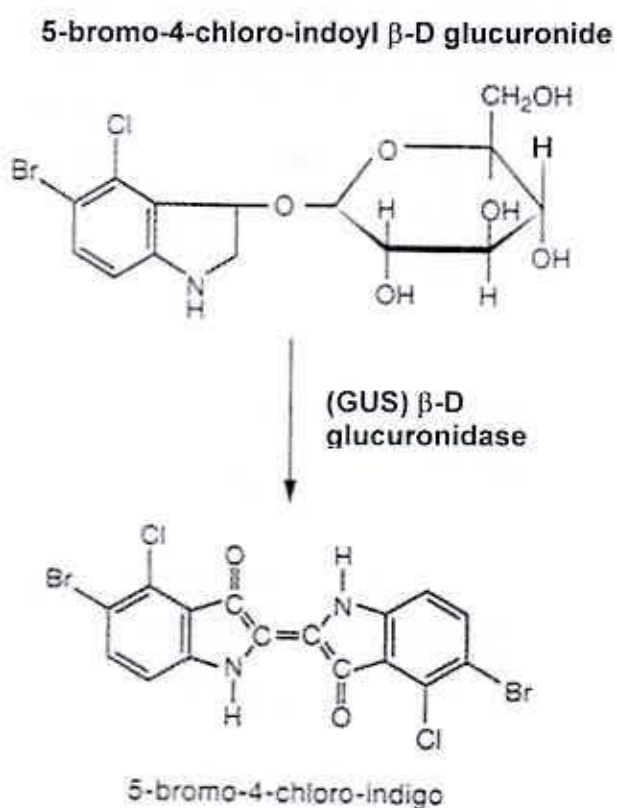
- Following 24 hours co-cultivation, the explants were transferred to regeneration medium consisting of MS medium supplemented with 3.0 mg/l BAP, 0.5 mg/l IAA and 500  $\mu\text{g/ml}$  cefotaxime.
- After 6-7 weeks, explants regenerated shoots were transferred to hormone free MS media with 250  $\mu\text{g/ml}$  cefotaxime.
- Amount of cefotaxime become half of its previous in every subculturing.

### 3.8.2.7. GUS ( $\beta$ -glucuronidase) Histochemical Assay

From each batch of explants following each transformation experiment, randomly selected tissues regenerated on nutrient media were examined for GUS histochemical assay. For this experiment, regenerated shoot tissues were immersed in X-gluc (5-bromo-4-chloro-3-indoyl glucuronide) solution and were incubated at  $37^{\circ}\text{C}$  overnight. A characteristic blue colour would be the expression of GUS ( $\beta$ -glucuronidase) gene in the plant tissue. Proper control for GUS histochemical assay was done with the explants having no *Agrobacterium* infection.

### 3.8.2.7.1. Preparation of GUS Assay Solution

Various  $\beta$ -glucuronic acid substrates are available for detection of GUS expression *in vivo* or *in vitro*. All of these substrates contain the sugar D-glucopyranosiduronic acid attached by glucosidic linkage to a hydroxyl group of a chromogenic, fluorogenic, or other detectable molecules (Figure 4). The preferred substrate for GUS detection is 5-bromo-4-chloro-3-indoyl- $\beta$ -D-glucuronide or X-gluc. This colorless substrate has high extinction coefficient (making it readily detectable at low concentrations) and aqueous insolubility of the final cleavage product, dichloro-dibromo-indigo (ClBr-indigo). The GUS staining solution is composed of following chemicals with their concentration (Table 3).



**Figure 4.** Reaction catalyzed by  $\beta$ -D glucuronidase

**Table 3. Component of GUS staining solution**

| <b>Components</b>   | <b>Amount/10 ml</b> |
|---|---------------------|
| X-gluc (solvent:DMSO)   | 8.89 mg             |
| Chloramphenicol   | 1 mg                |
| NaH <sub>2</sub> PO <sub>4</sub>                                      | 119.8 mg            |
| Triton X (10%)  | 100µl               |
| Methanol  | 2ml                 |
| pH was adjusted 7.0-8.0 by adding up to 10 ml buffer solution (pH-10) |                     |

### **3.8.2.7.2. Preparation of 10 ml GUS Staining Solution**

For the Preparation of 10 ml GUS Staining Solution the following steps were followed:

- All necessary glassware were autoclaved
- The 8.89 mg X-gluc were weighted with the help of a digital balance and due to high molecular weight; it was taken carefully in a very minute amount.
- Few drops of DMSO (Dimethyl Sulphoxide) were taken in a beaker and X-gluc was added.
- Beaker with DMSO was gently shaken until all of the X-gluc was dissolved.
- 200 µl of Chloramphenicol was added into the beaker.
- 10% Triton X was prepared by taking 20µl of Triton X into 200 µl of distilled water. (The Triton X first appeared as a gel like semisolid substance, but soon dissolved upon shaking gently. Then 100ml Triton X was added to the X-gluc solution.)
- 2 ml of methanol was added to the solution and gently mixed.
- pH of this solution was adjusted to 7.15 by adding pH buffer 10 solutions. It was noted that nearly 7 ml of buffer solution was need to adjust pH.



### **3.9. Culture Environment**

All cultures were grown in an air-conditioned growth room illuminated by 40W white fluorescent tube light. The temperature of the culture room was maintained at  $28 \pm 2$  °C.

### **3.10. Transferred to the Rooting Media**

Regenerated transferred shoots were subcultured to hormone free MS medium containing cefotaxime (half of the previous medium).

### **3.11. Subculturing**

Before transferring to the soil, regenerated shoots were subjected to subculturing in hormone free MS media. In each case, amount of cefotaxime reduced half of its previous concentration. Subculturing also done for shoot multiplication through cutting.

### **3.12. Transferred to Soil**

After 4-5 times subculturing, shoots were transferred to sterilize soil. After hardening, regenerated shoots were transferred to normal soil.

## **Preparation of Antibiotics (Kanamycin and Cefotaxime)**

### **Stock solution of Kanamycin**

- Required concentration of kanamycin 50 µg/ml
- Concentration of stock solution 50 µg/ml
- Volume of stock solution 10 ml
- So, 10 ml solution contains  $50 \mu\text{g} \times 10 = 500 \mu\text{g}$  Kanamycin.

### **Steps:**

- 500 µg Kanamycin was weighed by balance
- It was kept in 100 ml conical flask
- 10 ml sterile distilled water was added to the flask and dissolved by hand shaking.
- Filter sterilization was done with disposable millipore filter (0.22 µm pore size and syringe).
- Distributed by 1 ml with the help of micropipette to five sterilized eppendorf tubes and stored in dark condition at 4°C temperature,
- 1 ml stock solution contains 50 µg kanamycin.

### **Stock Solution of Cefotaxime**

- Required concentration of Cefotaxime 500 µg/l
- Concentration of stock solution 500µg/ml
- Volume of stock solution 10 ml
- So. 10 ml solution contains  $500 \text{ mg} \times 10 = 5000 \text{ µg}$  Cefotaxime.

### **Steps:**

- 1 g Cefotaxime in vial in powdery form was used.
- Sterile 10 ml syringe is used to transfer 5 ml sterile distilled water in the vial
- Hand shaking of the vial
- Stored in dark condition at deep freezer
- 1 ml stock contains 500µg Cefotaxime.

## **Infection of Kenaf Cotyledons with *Agrobacterium tumefaciens* Strain LBA 4404 / (Plasmid pBI121)**

- The infection medium of strain LBA4404 (plasmid pBI121) was distributed in a sterilized petri dish.
- Cotyledons with attached petioles were dipped for 1 minute.
- Prior to transfer of all explants to co-cultivation medium explants were blotted dry with sterile tissue papers for a short period of time to remove excess of bacterial suspension
- All the explants were maintained in co-cultivation media for 24 hours.
- Co-cultured conical flasks containing explants were placed under fluorescent illumination for 12 hour's alternate light and dark condition at  $28 \pm 2^{\circ}\text{C}$ .
- The intensity of light was maintained at 1500 lux.

### **3.13. Data Recording**

To investigate the effects of different treatments and responses of different varieties to callus induction and plantlet regeneration, data were collected from the different parameters as given below:

#### **3.13.1. Callus Induction**

#### **3.13.2. Days to Callus Initiation**

Generally callus initiation started after 5-8 days of incubation of explants. The number of callus initiated over a number of days was recorded.

### 3.13.3. Number of Calli/Conical Flask (Per cent Callus Induction)

The number of explants producing callus in each Petri dish was recorded. The percentage of callus induction was calculated on the basis of the number of explants placed and the total number of callus induced.

$$\text{Per cent callus induction} = \frac{\text{No. of explant produced calli}}{\text{No. of explant incubated}} \times 100$$

### 3.13.4. Days to Regeneration

Days required for regeneration was recorded at the time of initiation of shoots from the callus.

### 3.13.5. Number of Shoots / Callus

The number of calli producing plant-lets in each petridish was recorded. The percentage of callus induction was calculated on the basis of the number of explant placed and the total no. of callus induced.

$$\text{Per cent plant regnration} = \frac{\text{No. of calli produced shoot}}{\text{No. of explant cultured}} \times 100$$

### 3.13.6. Per cent GUS Positive

The number of cotyledons giving positive response to GUS histochemical assay was recorded. The percentage of GUS positive was calculated on the basis of the number of calluses assayed for GUS and the total number of calluses positive for GUS.

$$\text{Per cent GUS positive} = \frac{\text{No. of callus positive for GUS}}{\text{No. of calli assayed for GUS}} \times 100$$

### 3.13.7. Per cent Transgenic Plants


The percentage of transgenic plants was calculated based on the number of calli transferred to the selection and regeneration medium and the number of calli produced plantlets.

$$\text{Per cent plant transformation} = \frac{\text{No. of calli produced plantlets}}{\text{No. of inoculated explant}} \times 100$$

### 3.14. Statistical Analysis

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





**Chapter IV**  
**Results and Discussion**

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## CHAPTER IV RESULTS AND DISCUSSIONS

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Two experiments were conducted for the present research work. In the first experiment, *in vitro* plant regeneration of two kenaf varieties were conducted using various hormone concentrations. Influence of two different factors i.e. (a) effect of different age of explants and (b) effect of different BAP concentrations were observed. In the second experiment, *Agrobacterium*-mediated genetic transformation was carried out. The results of the experiments are described along with the discussion under following heads.

### **4.1. Experiment-1:** *In vitro* regeneration of two varieties of kenaf (*Hibiscus cannabinus*)

#### **4.1.1. Effect of Age of Explant**

*In vitro* seedlings were raised (Plate 1) and the cotyledons were placed on MS medium containing hormone in 250 ml conical flasks. Four (4) cotyledons were placed per flask and observed the response of different variables.

##### **4.1. 1.1. Effect of Varieties**

Two different varieties HC-2 and HC-95 were used for this experiment. following results Cotyledons with attached petioles of 6, 8, 10, 12, 14 days old were placed on conical flask containing MS medium and the responses of different variables like number of callus per flask, per cent callus induction, number of shoot per callus, number of callus producing shoot, per cent shoot regeneration, days to callus induction and days to shoot regeneration were measured.

It was shown (Table 4 and Table 5) that there was no significant difference between the varieties for number of callus per flask ( 2.1 for both HC-2 and HC-95), per cent callus induction (53.3% in HC-2 and 51.7% in HC-95), number of shoot per callus (1.7 for both HC-2 and HC-95), number of callus producing shoot (2.0 for HC-2 and 1.7 for HC-95), per cent shoot regeneration (45.0% for both HC-2 and HC-95), days to callus induction (6.0 for HC-2 and 5.9 for HC-95) and days to shoot regeneration (12.9 for both HC-2 and HC-95). It was clear that both the varieties had same effect on callusing, plant regeneration and days to callus induction and plant regeneration.

**Table 4. Effect of varieties on number of callus per flask, per cent callus induction, number of shoot per callus, number of callus producing shoot.**

| Variety | Number of callus/flask | % Callus induction | Number of shoot/callus | Number of callus producing shoot |
|---------|------------------------|--------------------|------------------------|----------------------------------|
| HC-2    | 2.1                    | 53.3               | 1.7                    | 2.0                              |
| HC-95   | 2.1                    | 51.7               | 1.7                    | 1.7                              |

**Table 5. Effect of varieties on per cent shoots regeneration, days to callus induction and days to shoot regeneration.**

| Variety | % Shoot regeneration | Days to callus induction | Days to shoot regeneration |
|---------|----------------------|--------------------------|----------------------------|
| HC-2    | 45.0                 | 6.0                      | 12.9                       |
| HC-95   | 45.0                 | 5.9                      | 12.9                       |





**Plate 1. *In vitro* Seedling rising of kenaf**

#### **4.1. 1.2. Effect of Age of Explant on Plant Regeneration**

##### **4.1. 1.2.1. Callus Induction**

Callus induction followed by plant regeneration was the first step of the experiment. *In vitro* callus induction depends on a number of factors including proper concentration of growth regulators used, age of explant (i.e, cotyledon) and the response of varieties. Cotyledons with attached petioles of two kenaf varieties (HC-2 and HC-95) with five age limits (6, 8,10,12,14 days) were used to observe their callusing response.





**Plate 2. Explant culture**



**Plate 3. Callus induction**



**Plate 4. Six days old explant failed to produce callus**

Cotyledons of different ages with attached petioles were placed on MS medium with hormone in conical flasks (Plate 2 and Plate 3) and responses were observed carefully. It was observed (Table 6) that the effect of 5 different age limits were statistically different (appendix I) for number of callus per flask and per cent callus induction. The maximum number of callus (3.5) was observed when 10 days old seedlings were used. Six (6) days old aged seedlings produced no callus (Plate 4). Both 8 and 12 days old seedlings produced more than 50% callus. 10 days old seedlings was the best for callusing.

**Table 6. Effect of age of explant on number of callus per flask, per cent callus induction.**

| <b>Age of explant (days)</b> | <b>Number of calli/flask</b> | <b>% Callus induction</b> |
|------------------------------|------------------------------|---------------------------|
| 6                            | 0.0d                         | 0.0d                      |
| 8                            | 2.3bc                        | 58.3bc                    |
| 10                           | 3.5a                         | 87.5a                     |
| 12                           | 2.7b                         | 66.7b                     |
| 14                           | 2.0c                         | 50.0c                     |

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

#### 4.1.1.2.2. Plant Regeneration

It was observed (Plate 5) that the highest number of shoot per callus (3.2), callus producing shoot (3.2) and per cent shoot regeneration (79.2) was found in case of 10 days old explant. Minimum no. of shoot per callus (1.2) and per cent shoot regeneration (33.3%) was observed in case of 14 days old explant (Table 7). It was clear that significant statistically difference (appendices I and II) present for different age of explants. Among the explants 6 days old cotyledon produced no callus (Plate 4).

**Table 7. Effect of age of explant on number of shoot per callus, number of callus producing shoots and per cent shoot regeneration**

| Age of explant | Number of shoot per callus | Number of callus producing shoot | Percent shoot regeneration. |
|----------------|----------------------------|----------------------------------|-----------------------------|
| 6              | 0.0e                       | 0.3c                             | 0.0d                        |
| 8              | 1.8c                       | 2.3ab                            | 54.2b                       |
| 10             | 3.2a                       | 3.2a                             | 79.2a                       |
| 12             | 2.3b                       | 2.2b                             | 58.3b                       |
| 14             | 1.2d                       | 1.2c                             | 33.3c                       |

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



Plate 5. Shoot produced from callus a) 8 days old explant, b) 12 days old explant, c) 10 days old explant



#### 4.1.1.2.3. Days to Callus Induction and Plant Regeneration

It was showed that minimum days required for callus induction was 6.2 days and shoot regeneration was 14.5 days (Table 8). Both of the results were found in case of 10 days old explant. Maximum days for callus induction were 8.7. It was observed in case 14 days old explant. Maximum days to shoot regeneration was 17.3 days and it was observed in case of both 8 and 10 days old explant.

The mean value of different age of explants showed statistically difference (Appendix II) for days to callus induction and days to shoot regeneration.

**Table 8. Effect of age of explant on days to callus induction and days to shoot regeneration**

| Age of explant | Days to callus induction | Days to shoot regeneration |
|----------------|--------------------------|----------------------------|
| 6              | 0.0d                     | 0.0c                       |
| 8              | 7.8b                     | 17.3a                      |
| 10             | 6.2c                     | 14.5b                      |
| 12             | 7.2b                     | 15.3b                      |
| 14             | 8.7a                     | 17.3a                      |

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

#### 4.1.1.3. Combined Effect of Variety and Different Ages of Explant on Plant Regeneration

Analysis was made to know if there was any variation due to the interaction among different varieties and age (Table 9, Table 10 & Table 11) on number of callus per flask, per cent callus induction, number of shoot per callus, number of callus producing shoot and percent shoot regeneration, days to callus induction and shoot regeneration. The highest number of calli/flask was 3.7 at 10 days old HC-2 explant and 3.3 at HC-95. The lowest number of callus observed at 14 days old cotyledon in case of both varieties and it was 2.0. The highest 91.7% callus observed at HC-2 when 10 days old explants were used. The lowest 50% callusing in case of 14 days old explant in both varieties (Table 9).

**Table 9. Combined effect of variety and age of explant on number of callus per flask, per cent callus induction.**

| Variety | Age of explant (days) | Number of calli/flask | % Calli induction |
|---------|-----------------------|-----------------------|-------------------|
| HC-2    | 6                     | 0.0 c                 | 0.0 c             |
|         | 8                     | 2.3 b                 | 58.3 b            |
|         | 10                    | 3.7 a                 | 91.7 a            |
|         | 12                    | 2.7 b                 | 66.7 b            |
|         | 14                    | 2.0 b                 | 50.0 b            |
| HC-95   | 6                     | 0.0 c                 | 0.0 c             |
|         | 8                     | 2.3 b                 | 58.3 b            |
|         | 10                    | 3.3 a                 | 83.3 a            |
|         | 12                    | 2.7 ab                | 66.7 ab           |
|         | 14                    | 2.0 b                 | 50.0 b            |

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



The combined effect of variety and age of explant on number of shoot per callus, number of callus producing shoot and percent shoot regeneration response were also observed (Table 10). The highest number of shoot per callus was observed 3.3 at 10 days old explants of HC-2 variety and the lowest number of shoot per callus was observed 1.0 at 14 days old cotyledons of HC-95 variety.

**Table 10. Combined effect of varieties and age of explant on number of shoot per callus, number of callus producing shoot and percent shoot regeneration**

| variety | Age of explant (days) | Number of shoot/callus | Number of shoot producing callus | % Shoot regeneration |
|---------|-----------------------|------------------------|----------------------------------|----------------------|
| HC-2    | 6                     | 0.0 d                  | 0.0 c                            | 0.0 d                |
|         | 8                     | 1.7 bc                 | 2.3 ab                           | 50.0 bc              |
|         | 10                    | 3.3 a                  | 3.3 a                            | 83.3 a               |
|         | 12                    | 2.3 b                  | 2.7 ab                           | 58.3 b               |
|         | 14                    | 1.3 c                  | 1.7 b                            | 33.3 c               |
| HC-95   | 6                     | 0.0 d                  | 0.0 b                            | 0.0 c                |
|         | 8                     | 2.0 b                  | 2.3 a                            | 58.3 a               |
|         | 10                    | 3.0 a                  | 3.0 a                            | 75.0 a               |
|         | 12                    | 2.3 ab                 | 1.7 ab                           | 58.3 a               |
|         | 14                    | 1.0 c                  | 0.7 b                            | 33.3 c               |

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

The highest (3.3) number of callus produced shoot by 10 days old HC-2 variety and the lowest number of callus (0.7) was produced by 14 days old explants. The Highest 83.3% shoot regeneration occurred successfully at 10 days old HC-2 variety and the lowest 33.3% at 14 days old both HC-2 and HC-95 varieties. No regeneration occurred at any variety of 6 days aged explants. Minimum days to callus induction were 6.3 in HC-2 variety and 6.0 in HC-95. Minimum days to shoot regeneration required 14.7 for HC-2 and 14.3 for HC-95 kenaf variety (Table 11).

**Table 11. Combined effect of varieties and age of explant on days to callus induction and shoot regeneration**

| Variety | Age of explant(days) | Days to callus induction | Days to shoot regeneration |
|---------|----------------------|--------------------------|----------------------------|
| HC-2    | 6                    | 0.0 d                    | 0.0 c                      |
|         | 8                    | 7.7 ab                   | 17.7 a                     |
|         | 10                   | 6.3 c                    | 14.7 b                     |
|         | 12                   | 7.3 bc                   | 15.0 b                     |
|         | 14                   | 8.7 a                    | 17.3 a                     |
| HC-95   | 6                    | 0.0 d                    | 0.0 d                      |
|         | 8                    | 8.0 ab                   | 17.0 ab                    |
|         | 10                   | 6.0c                     | 14.3 c                     |
|         | 12                   | 7.0 bc                   | 15.7 bc                    |
|         | 14                   | 8.7a                     | 17.3 a                     |

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

## 4.1.2. Effect of BAP Concentration

### 4.1.2.1. Effect of Variety

It was clear that there was no significant difference between the varieties for callus induction and days to callus induction and plant regeneration due to BAP concentration (Table 12 and Table 13).

**Table 12. Effect of varieties on number of callus per flask, per cent callus induction, days to shoot regeneration and number of callus producing shoot.**

| Variety | Number of callus/ flask | % Callus induction | No. of shoot/ callus | No. of callus producing shoot |
|---------|-------------------------|--------------------|----------------------|-------------------------------|
| HC-2    | 1.6                     | 39.3               | 1.5                  | 1.3                           |
| HC-95   | 1.7                     | 41.7               | 1.4                  | 1.3                           |

**Table 13. Effects of variety on per cent shoot regeneration, days to callus induction and days to shoot regeneration.**

| Variety | % Shoot regeneration | Days to callus induction | Days to shoot regeneration |
|---------|----------------------|--------------------------|----------------------------|
| HC-2    | 34.5                 | 4.9                      | 10.6                       |
| HC-95   | 33.3                 | 5.0                      | 11.0                       |

## 4.1.2.2. Effect of BAP Concentration on Regeneration

### 4.1.2.2.1. Callus Induction

Explants were placed on flask with MS medium containing different BAP concentration (0, 1, 2, 3, 4, 5, 6 mg/l) and observed that maximum no. of callus /flask (3.7) and per cent callus induction (91.7%) at 3mg/l BAP concentration. It was clear that significantly different response of BAP concentration (Appendix-III) present on number of callus per flask, per cent callus induction (Table 14).

**Table 14. Effect of BAP concentration on number of callus per flask, per cent callus induction**

| BAP concentration | Number of callus /flask | % Callus induction |
|-------------------|-------------------------|--------------------|
| 0                 | 0.0 e                   | 0.0 e              |
| 1                 | 0.0 e                   | 0.0 e              |
| 2                 | 1.2 d                   | 29.2 d             |
| 3                 | 3.7 a                   | 91.7 a             |
| 4                 | 3.0 b                   | 75.0 b             |
| 5                 | 2.2 c                   | 54.2 c             |
| 6                 | 1.3 d                   | 33.3 d             |

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



#### 4.1.2.2.2. Regeneration

Different BAP concentration had significantly different (Appendices-III and Appendices-III) response on number of shoot per callus, number of callus producing shoot and percent shoot regeneration. The highest number of shoot per callus was observed 3.5 at MS medium supplemented with 3mg/l BAP concentration and lowest 0.8 at 2 mg/l BAP concentration (Table 15). Maximum number of callus produced shoot (3.5) at MS medium with 3mg/l BAP concentration and minimum (0.7) at 2 mg/l BAP concentration. The highest 87.5% shoot regenerated at 3 mg/l BAP concentration and the lowest (16.7%) at 2 mg/l BAP condition. No callusing and regeneration occurred at MS medium containing 0 and 1 mg/l BAP.

**Table 15. Effect of BAP concentration on number of shoot per callus, number of callus producing shoot and percent shoot regeneration.**

| BAP concentration | Number of shoot per callus | Number of callus producing shoot | % Shoot regeneration |
|-------------------|----------------------------|----------------------------------|----------------------|
| 0                 | 0.0 e                      | 0.0 e                            | 0.0 e                |
| 1                 | 0.0 e                      | 0.0 e                            | 0.0 e                |
| 2                 | 0.8 d                      | 0.7 d                            | 16.7 d               |
| 3                 | 3.5 a                      | 3.5 a                            | 87.5 a               |
| 4                 | 2.8 b                      | 2.5 b                            | 66.7 b               |
| 5                 | 2.0 c                      | 1.7 c                            | 41.7 n               |
| 6                 | 1.2 d                      | 1.0 d                            | 25.0 d               |

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

#### 4.1.2.2.3. Days to Callus Induction and Plant Regeneration

Significant differences also observed in case of days to callus initiation and days to shoot regeneration due to different concentration of BAP (Appendix-IV). Minimum 6.3 days required for callusing and 13.7 days for shoot regeneration when 2 mg/l BAP was used when BAP concentration was 3 mg/l, the highest 7.5 days for callusing 15.7 days for shooting required (Table 16).

**Table 16. Effect of BAP concentration on days to callus induction and days to shoot regeneration**

| <b>BAP concentration</b> | <b>Days to callus induction</b> | <b>Days to shoot regeneration</b> |
|--------------------------|---------------------------------|-----------------------------------|
| 0                        | 0.0 b                           | 0.0 b                             |
| 1                        | 0.0 b                           | 0.0 b                             |
| 2                        | 6.3 a                           | 13.7 a                            |
| 3                        | 7.5 a                           | 15.7 a                            |
| 4                        | 7.3 a                           | 15.7 a                            |
| 5                        | 6.7 a                           | 15.5 a                            |
| 6                        | 6.5 a                           | 14.8 a                            |

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

#### 4.1.2.3. Combined Effect of Variety and Different Concentration BAP on Regeneration

It was showed that both the varieties had more or less same effect incase of callusing due to combine effect of variety and BAP concentration, The maximum no. of calli/flask was observed 3.7 incase of 3mg/l BAP conc. in case of both HC-2 & HC-95 varieties (Table 17). No callus was found in case of BAP concentration 0 and 1 mg/l.

**Table 17. Combined effect of varieties and BAP concentration on number of callus per flask, per cent callus induction.**

| Variety | BAP concentration (mg/l) | IAA concentration (mg/l) | Number of callus/flask | % Callus induction |
|---------|--------------------------|--------------------------|------------------------|--------------------|
| HC-2    | 0                        | 0.05                     | 0.0 e                  | 0.0 e              |
|         | 1                        | 0.05                     | 0.0 e                  | 0.0 e              |
|         | 2                        | 0.05                     | 1.0 d                  | 25.0 d             |
|         | 3                        | 0.05                     | 3.7 a                  | 91.7 a             |
|         | 4                        | 0.05                     | 3.0 b                  | 75.0 b             |
|         | 5                        | 0.05                     | 2.3 c                  | 58.3 c             |
|         | 6                        | 0.05                     | 1.0 d                  | 25.0 d             |
| HC-95   | 0                        | 0.05                     | 0.0 e                  | 0.0 e              |
|         | 1                        | 0.05                     | 0.0 e                  | 0.0 e              |
|         | 2                        | 0.05                     | 1.3 d                  | 33.3 d             |
|         | 3                        | 0.05                     | 3.7 a                  | 91.7 a             |
|         | 4                        | 0.05                     | 3.0 b                  | 75.0 b             |
|         | 5                        | 0.05                     | 2.0 c                  | 50 c               |
|         | 6                        | 0.05                     | 1.7 cd                 | 41.7 d             |

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

The highest per centage of callusing was 91.7% in both two varieties at BAP level 3mg/l. Highest number of shoot per callus( 3.7), number of callus producing shoot (3.7) and the highest per cent shoot regeneration also observed in case of 3mg/l BAP concentration (Table 18) in two kenaf varieties (HC-2 and HC-95). No callus was observed in case of 0 and 1 mg/l BAP concentration in both cases.

**Table 18. Combined effect of varieties and BAP concentration on number of shoot per callus, number of callus producing shoot and per cent shoot regeneration.**

| Variety | BAP concentration (mg/l) | IAA concentration (mg/l) | Number of shoot/callus | Number of callus producing shoot | % Shoot regeneration |
|---------|--------------------------|--------------------------|------------------------|----------------------------------|----------------------|
| HC-2    | 0                        | 0.05                     | 0.0 d                  | 0.0 e                            | 0.0 e                |
|         | 1                        | 0.05                     | 0.0 d                  | 0.0 e                            | 0.0 e                |
|         | 2                        | 0.05                     | 1.0 c                  | 0.7 de                           | 16.7 cd              |
|         | 3                        | 0.05                     | 3.7 a                  | 3.7 a                            | 91.7 a               |
|         | 4                        | 0.05                     | 3.0 a                  | 2.3 b                            | 66.7 b               |
|         | 5                        | 0.05                     | 2.0 b                  | 1.7 bc                           | 41.7 c               |
|         | 6                        | 0.05                     | 1.0 c                  | 1.0 cd                           | 25.0 bc              |
| HC-95   | 0                        | 0.05                     | 0.0 e                  | 0.0 d                            | 0.0 e                |
|         | 1                        | 0.05                     | 0.0 e                  | 0.0 d                            | 0.0 e                |
|         | 2                        | 0.05                     | 0.7 de                 | 0.7 cd                           | 16.7 cd              |
|         | 3                        | 0.05                     | 3.3 a                  | 3.3 a                            | 83.3 a               |
|         | 4                        | 0.05                     | 2.7 ab                 | 2.7 a                            | 66.7 a               |
|         | 5                        | 0.05                     | 2.0 bc                 | 1.7 b                            | 41.7 b               |
|         | 6                        | 0.05                     | 1.3 cd                 | 1.0 bc                           | 25.0 bc              |

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



Due to combined effect of BAP concentration and variety, days to callus induction and shoot regeneration was more or less similar in both variety.. Maximum days to callusing was 7.3 (HC-2) and 8.0 (HC-95). Minimum days to callusing was 6.3 in both variety and the minimum days required for shoot regeneration was 13.3 in HC-2 and 14.0 in HC-95 (Table 19).

**Table 19. Combined effect of varieties and BAP concentrations on days to callus induction and days to shoot regeneration.**

| Variety | BAP concentration (mg/l) | IAA concentration (mg/l) | Days to callus induction | Days to shoot regeneration |
|---------|--------------------------|--------------------------|--------------------------|----------------------------|
| HC-2    | 0                        | 0.05                     | 0.0 b                    | 0.0 b                      |
|         | 1                        | 0.05                     | 0.0 b                    | 0.0 b                      |
|         | 2                        | 0.05                     | 6.3 a                    | 13.3 a                     |
|         | 3                        | 0.05                     | 7.0 a                    | 15.3 a                     |
|         | 4                        | 0.05                     | 7.3 a                    | 15.7 a                     |
|         | 5                        | 0.05                     | 6.7 a                    | 15.7 a                     |
|         | 6                        | 0.05                     | 6.7 a                    | 14.0 a                     |
| HC-95   | 0                        | 0.05                     | 0.0 b                    | 0.0 b                      |
|         | 1                        | 0.05                     | 0.0 b                    | 0.0 b                      |
|         | 2                        | 0.05                     | 6.3 a                    | 14.0 a                     |
|         | 3                        | 0.05                     | 8.0 a                    | 16.0 a                     |
|         | 4                        | 0.05                     | 7.3 a                    | 15.7 a                     |
|         | 5                        | 0.05                     | 6.7 a                    | 15.3 a                     |
|         | 6                        | 0.05                     | 6.3 a                    | 15.7 a                     |

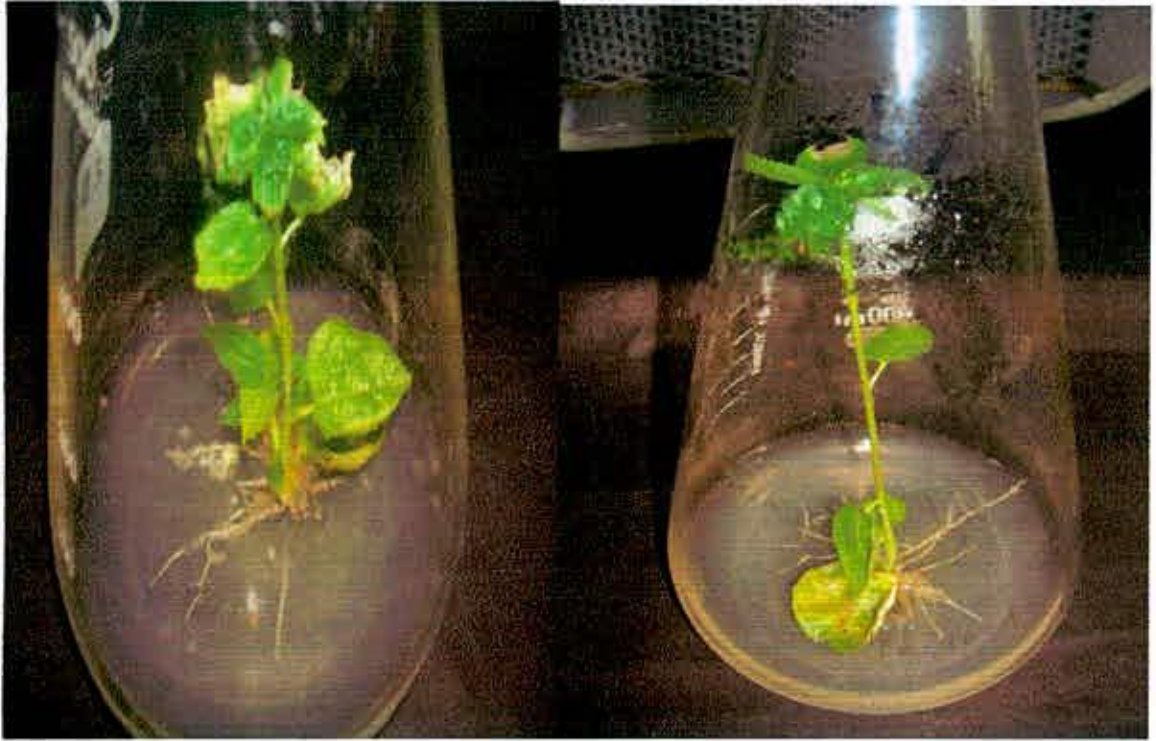
Figures followed by same letter in a column do not differ significantly by DMRT.

#### 4.1.2.4. Root Induction

The shoots regenerated from this experiment were transferred to MS medium without any hormone and all the shoots produced root (Plate 6 & Plate 7)



**Plate 6. Regenerated shoot transferred to the rooting medium**



**Plate 7. Regenerated plantlets Produced root**

#### **4.1.2.5. Transferred to Soil**

After root production, regenerants were transferred to sterilized soil. After subsequent hardening, plantlets were transferred to normal soil (Plate 8, Plate 9 & Plate 10).





**Plate 8. Regenerated shoot Transferred to sterilized soil**



**Plate 9. Hardening of regenerated plant**



**Plate 10. Regenerated shoot transferred to normal soil**

## **4.2. Experiment - 2. *Agrobacterium* - Mediated Genetic Transformation in Kenaf**

Genetic transformation is a powerful and important tool for crop improvement. It can be used in plant breeding program for kenaf improvement as it permits access to an unlimited gene pool through the transfer of desirable genes from any source. However, an efficient and reproducible transformation protocol is required for successful transfer of desirable gene. Therefore, in the present study, investigations were made to generate transgenic plant from two varieties of *Hibiscus cannabinus* through *Agrobacterium* mediated genetic transformation using the cotyledons with attached petioles as explants.

In the previous study on plant regeneration in kenaf varieties, it was evident that the cotyledons with attached petioles of two varieties have the inherent capability of plant regeneration. For this reason, cotyledons with attached petioles were subjected to *Agrobacterium* mediated transformation experiment using LBA 4404 strain. As the MS media supplemented with 0.5 mg/l IAA and 3.0 mg/l BAP showed the best shoot regeneration, this hormone combination was used for regeneration of putative transgenic plants.

### **4.2.1. Selection of Putative Transformed Shoot**

All the explants were transferred to kanamycin containing MS medium for selecting transformed callus from various explants. Following infection and co-cultivation explants were transferred to MS medium supplemented with 3.0 mg/l BAP, 0.5 mg/l IAA and 50 mg/l kanamycin (Plate11, Plate12 & Plate 13).



Plate 11. Streak culture of *Agrobacterium tumefaciens* strain LBA 4404 plasmid pB1121

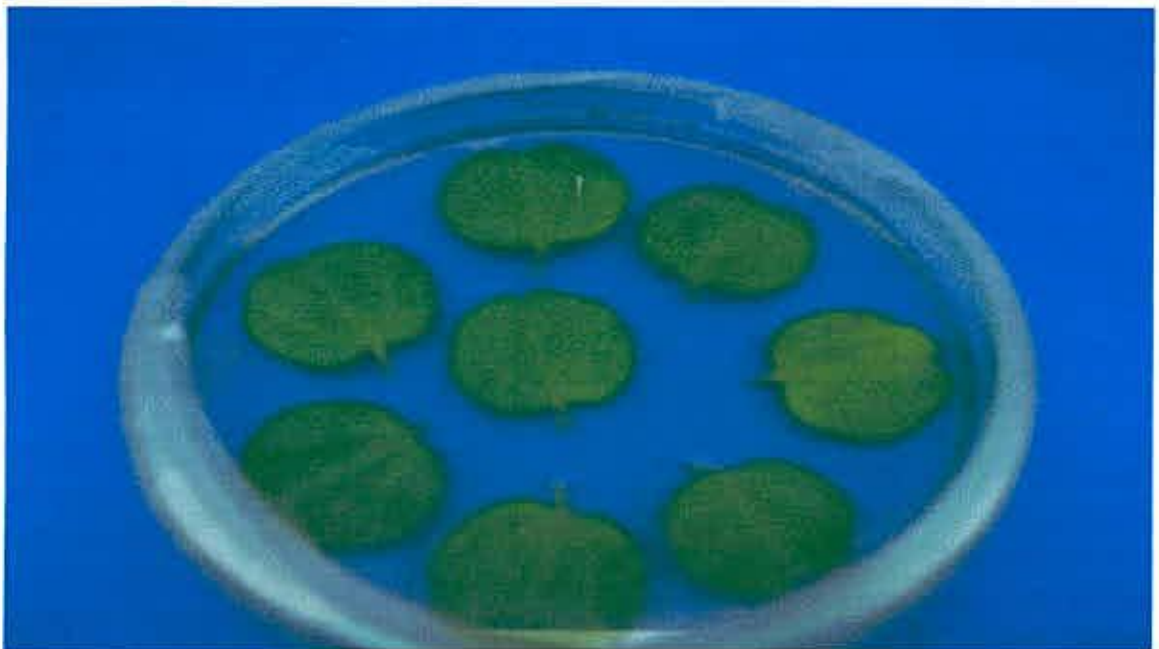
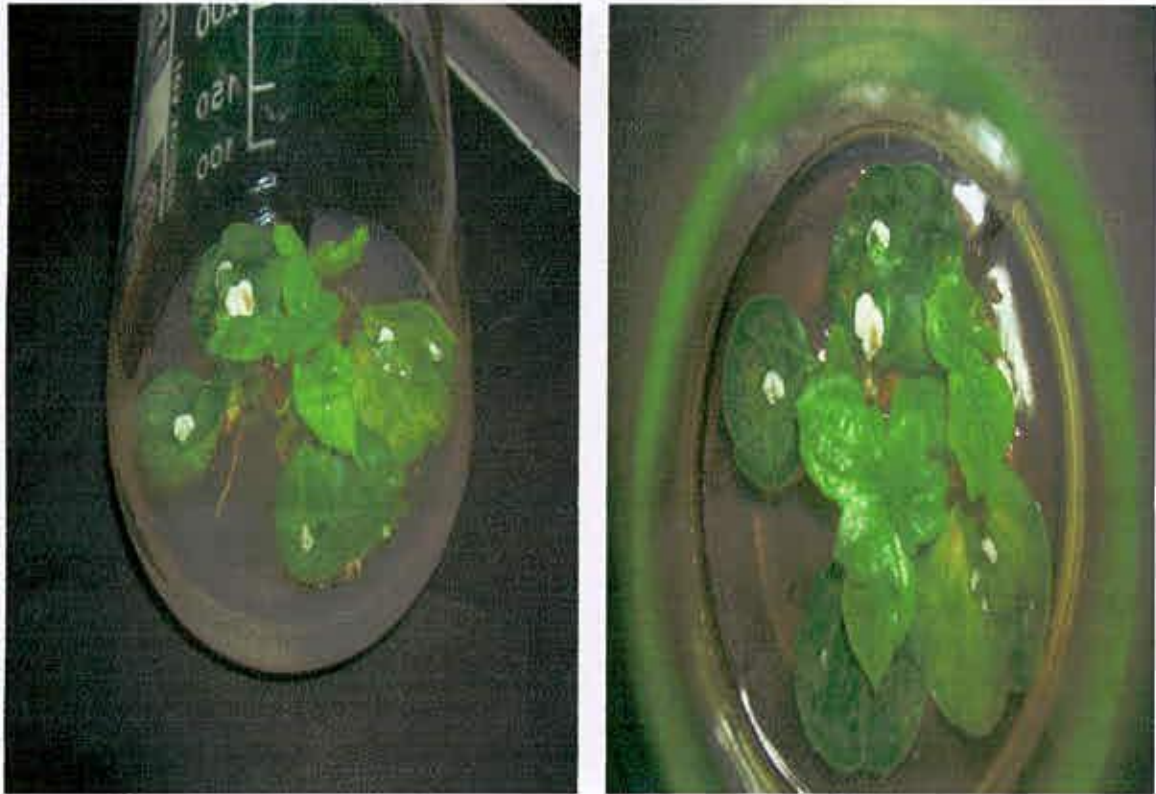


Plate 12. Co-culture of explant with *Agrobacterium* strain pBI121





**Plate 13. Transgenic shoot regeneration in nutrient medium.**

Non-transgenic shoots became albino and ultimately died after certain period. The highest number (85%) of kanamycin resistant shoots was found in case of cotyledon /explants of variety HC-2 (Table 20 & Plate 14).

**Table 20. Selection of explant (cotyledon) using kanamycin**

| Variety | Explant   | Number of explant set for selection | Number of explant survived and regenerated | % Regeneration |
|---------|-----------|-------------------------------------|--|----------------|
| HC-2    | Cotyledon | 100                                 | 85   | 85             |
| HC-95   | Cotyledon | 100                                 | 83   | 83             |



**Plate 14. Putative transformed shoot**

#### 4.2.2. Determination of Transformation Ability of Two Kenaf Varieties

Following infection with *Agrobacterium*, explants were transferred to culture medium. (Regeneration medium) containing 0.5mg/l of IAA +3.0mg/l BAP+ 250 µg/ml cefotexme. Transformation ability of explants tissue was checked through histochemical assay of GUS reporter genes. Transient GUS assay was performed for 72 hours co-cultivated explants and for the regenerated shoots survived in selection medium. Tissues from randomly selected 20 explants of each type were taken for the histochemical assay. Conspicuous GUS positive (blue color) regions were detected in the thick section of regenerated shoots resistant to kanamycin.

**Table 21. Influence of explants on transformation as assessed through GUS histochemical assay**

| Varieties | Explants  | Number of explant infected | Number of explants assyed | Number of GUS positive explants | % GUS positive explants |
|-----------|-----------|----------------------------|---------------------------|---------------------------------|-------------------------|
| HC-2      | cotyledon | 100                        | 20                        | 18                              | 90                      |
| HC-95     | cotyledon | 100                        | 20                        | 17                              | 85                      |

After GUS histochemical assay, it was found that both of the varieties showed positive responses towards transformation (Table 21). Cotyledon-derived shoots of both of the varieties showed higher percentage of GUS expression (90.0% HC-2 and 85.0% HC-95).





**Plate 15. Thick stem section showing GUS positive tissues of cotyledons derived shoot (deep bluish zone in the circle) of variety HC-2**



**Plate 16. GUS positive tissues showing by circle in thick stem section of variety HC-2**

### **4.2.3. Transfer of Putative Transformed Shoots in Rooting Medium**

Survived transgenic shoots were transferred to hormone free MS medium but supplemented with cefotaxime (250 µg/ml) for shoot multiplication and root initiation (Plate 17). Subculturing was done after each four weeks. Amount of cefotaxime become half of its previous in each subculturing. Regeneration was found from variety HC-2 explant was 85% (Table 21).



**Plate 17. Shoot multiplication of putative transformed plantlet**

#### 4.2.4. Putative Transformed Shoots Transferred to Soil

After root production, transgenic plantlets were subjected to hardening and then transferred to soil successfully and obtained some transgenic seed also (Plate 18, Plate 19 & Plate 20, Plate 21, Plate 22, Plate 23, Plate 24 & Plate 25).



Plate 18. Transgenic seedling transferred to sterilize soil





**Plate 19. Hardening of transgenic seedlings**



**Plate 20. Transgenic seedlings Transferred to normal soil**







**Plate 21. The divided shape leaf of HC-95**



**Plate 22. The entire leaf shape of the variety HC-2**




**Plate 23. The kenaf flower.**



**Plate 24. Ripen transformed fruites**



**Plate 25. Transformed seeds**



**Chapter V**  
**Summary and Conclusion**

## CHAPTER V SUMMARY AND CONCLUSION

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Two different sets of experiments were conducted at the Department of Cytogenetics, Genetic Resources and Seed Division, Bangladesh Jute Research Institute, Dhaka during the period of January 2007 to November 2007. In the first experiment, a detailed investigation was carried out to study the callus induction ability and subsequent plant regeneration protocol for two varieties of kenaf (var. HC-2 and HC-95) using cotyledons with attached petioles as explants. In the second experiment, investigations were carried out to study *Agrobacterium* mediated genetic transformation.

In the first experiment, two different investigations were made to show the effect of different age of explants and BAP concentration on regeneration. Explants (cotyledons with attached petioles) of different ages (6, 8, 10, 12 and 14 days old) were cultured on MS medium to induce callus and plant regeneration. A wide range of variation was observed among the variables due to age differences. The range of callus induction was varied from 50.0% to 91.7%. The highest callus production (91.7%) was found in the variety HC-2 when 10 days old cotyledons were used and the lowest (50%) was found in both the varieties (HC-2 and HC-95) when 14 days old explants were used. No callusing observed when 6 days old explants were used.

Observations were also made for plants regeneration and large variation was also found. The ranges varied from 33.3% to 85.3%. The maximum shoot regeneration (85.3%) observed in HC-2 variety when 10 days old explants were used and the minimum (33.3%) was in both HC-2 and HC-95 variety when 14 days aged explants were used.

Both the varieties (HC-2 and HC-95) were cultured on MS medium supplemented with different concentration of BAP (0, 1, 2, 3, 4, 5 and 6 mg/l) and a constant IAA (0.5mg/l) concentration to induce callus and shoot regeneration. No callus induction and shoot regeneration was observed in case

of MS medium with 0.0 mg/l BAP and 1.0 mg/l BAP conditions. A wide range of variations in callus induction and shoot regeneration ability was observed by the varieties in media with different BAP concentration. The range of callus induction was 25.0 % to 91.7 % and shoot regeneration 16.7% to 91.7%. The highest callus induction (91.7%) was found in both HC-2 and HC-95 varieties when MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l IAA and the highest shoot regeneration (91.7%) in HC-2 variety. The lowest callus induction (25.0%) was observed in HC-2 and shoot production (16.7%) was found in both HC-2 and HC-95 when 2.0 mg/l BAP and 0.5 mg/l IAA was used in culture medium.

GUS histochemical assay was performed for the co-cultivated explants of kenaf varieties (shoots) regenerated on kanamycin for the detection of transgenic plants. *Agrobacterium tumefaciens* strain LBA4404 contains the genes for kanamycin resistance was identified as GUS expression. GUS expression (GUS gene) produced blue colour with X-gluc through GUS histochemical assay. Therefore, the presence of this blue colour gave a preliminary indication of GUS gene transfer from bacterial plasmid into the plant cells. The result of GUS assay varied according to varieties. Between the varieties response to GUS assay, H-2 showed 90.0% GUS positive whereas HC-95 was 85.0% positive for GUS assay.

Regeneration of plantlets (putative transformed plantlets) in kanamycin containing medium was the highest for the variety HC-2 (85.0%) followed by HC-95 (83.0%). Due to the time limit it was not possible to conduct PCR. For confirmation of gene transfer, further study like PCR, southern blotting and sequencing need to be done.

Finally, it can be concluded that efficient and reproducible protocol for plant regeneration of two kenaf varieties has been developed using cotyledons with attached petioles in the first experiment. Genetic transformation depends on efficient method of plant regeneration. For regeneration, 10 days old cotyledons placed on MS media having 3mg/l BAP concentration would be the best.

In second experiment, an efficient and reproducible protocol for transformation of kenaf varieties has been developed, which showed the integration of two marker genes (GUS and nptII).

Thus, in the future programme

- Economically important gene/genes like saline tolerant, drought resistant, disease and insect resistant genes would be transferred to the kenaf varieties against diseases and pests.
- Somaclonal variants that might be observed in these experiments would be used for further research.





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# Appendices



## APPENDICES

Appendix-I: Mean squares of explants growing callus, per cent callus induction number of shoot per callus

| Source of variation      | Degree of freedom | Mean square                  |                           |                            |
|--------------------------|-------------------|------------------------------|---------------------------|----------------------------|
|                          |                   | No of explant growing callus | Per cent callus induction | Number of shoot per callus |
| Factor A (variety)       | 1                 | 0.0333                       | 20.8                      | 0.0333                     |
| FactorB (age of explant) | 4                 | 10.1333**                    | 6333.3**                  | 8.6167**                   |
| Factor AxB               | 4                 | 0.0333                       | 20.8                      | 0.1167                     |
| error                    | 20                | 0.02000                      | 125.0                     | 0.1667                     |

CV = 21.3%

\*\* = Significant at 1% level



**Appendix-II: Mean squares of number of callus producing shoot, per cent shoot regeneration, days to callus induction and days to shoot regeneration.**

| Source of variation       | Degree of freedom | Mean square                      |                      |                          |                            |
|---------------------------|-------------------|----------------------------------|----------------------|--------------------------|----------------------------|
|                           |                   | Number of callus producing shoot | % Shoot regeneration | Days to callus induction | Days to shoot regeneration |
| Factor A (variety)        | 1                 | 0.0833                           | 0.0                  | 0.033                    | 0.03                       |
| Factor B (age of explant) | 4                 | 7.2500**                         | 5385.4**             | 71.783**                 | 321.30**                   |
| Factor AxB                | 4                 | 0.7500                           | 52.1                 | 0.117                    | 0.37                       |
| error                     | 20                | 0.5667                           | 187.5                | 0.367                    | 0.80                       |

CV = 21.3%

\*\* = Significant at 1% level

**Appendix-III: Mean squares of explants growing callus, per cent casllus induction, number of shoot per callus**

| Source of variation         | Degree of freedom | Mean squares                     |                            |                            |
|-----------------------------|-------------------|----------------------------------|----------------------------|----------------------------|
|                             |                   | Number of explant growing callus | per cent casllus induction | Number of shoot per callus |
| Factor A(variety)           | 1                 | 0.0952                           | 59.5                       | 0.0952                     |
| Factor B(BAP concentration) | 6                 | 11.9286**                        | 7455.4**                   | 11.0794**                  |
| FactorAxB                   | 6                 | 0.1508                           | 94.2                       | 0.0952                     |
| error                       | 28                | 0.1190                           | 74.4                       | 0.1905                     |

CV = 21.3%

\*\* = Significant at 1% level

**Appendix-IV: Mean squares of number of callus producing shoot, per cent shoot regeneration, days to callus induction and days to shoot regeneration.**

| Source of variation          | Degree of freedom | Mean square                      |                      |                          |                            |
|------------------------------|-------------------|----------------------------------|----------------------|--------------------------|----------------------------|
|                              |                   | Number of callus producing shoot | % Shoot regeneration | Days to callus induction | Days to shoot regeneration |
| Factor A (variety)           | 1                 | 0.000                            | 14.9                 | 0.095                    | 1.52                       |
| Factor B (BAP concentration) | 6                 | 10.2778**                        | 6681.5**             | 68.437**                 | 327.21**                   |
| Factor Ax B                  | 6                 | 0.0556                           | 14.9                 | 0.262                    | 0.69                       |
| error                        | 28                | 0.1905                           | 119.0                | 4.976                    | 21.02                      |

CV = 21.3%

\*\* = significant at 1% level

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সংযোজন নং... 40(৩) 38974  
তারিখ: ০৩/০৩/০৯