PLANT REGENERATION PROTOCOL IN KENAF (*Hibiscus canabinus* L.)

BY

AMENA SIDDIKA

REGISTRATION NO. 00512

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

MASTER OF SCIENCE

IN

GENETICS AND PLANT BREEDING

SEMESTER : JANUARY-JUNE 2007

Approved by:

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

.....
(Dr. Md. Sarowar Hossain)
Professor
SAU, Dhaka
Co-supervisor

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

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

CERTIFICATE

This is to certify that thesis entitled, ***PLANT REGENERATION PROTOCOL IN KENAF (Hibiscus canabinus L.)*** *" 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* ***AMENA SIDDIKA,*** *Registration No.00512 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

Dated: June, 2007
Place: Dhaka, Bangladesh

Asma Khatun

Dr. Asma khatun
CSO, BJRI, Dhaka
Supervisor

Dedicated to my beloved parents

LIST of ABBREVIATIONS

Abbreviations		Full word
%	:	Percentage
O.1N	:	O. l Normal
BAP	:	6-benzyl amino purine
CIP	:	International Potato Centre
DMRT	:	Duncan's Multiple Range Test
et al.	:	et alu=other people
etc.	:	et cetera (means and the rest)
Fig.	:	Figure
GA_3	:	Gibberellic acid
g	:	Gram
HC1	:	Hydrocloric acid
$HgC1_2$	:	Mercuric cloride
h	:	Hour (s)
IAA	:	Indole-3-acetic acid
IARI	:	Indian Agricultural Research Institute
IBA	:	Indole-3-butaric acid
ICRISAT	:	International Crop Research Institute for the Semi-arid Tropics
IRRI	:	International Rice Research Institute
J.	:	Journal
l	:	Litre
Lux	:	Unit of illumination
M	:	Molar or Manitol
mm	:	Minute (s)
mg	:	Milligram
mg/l	:	Milligram per litre
ml	:	Millititre

Abbreviations		Full word
ml/l	:	Millilitre per litre
MS	:	Murashige and Skoog (1962)
mW	:	Milliwatt(s)
N	:	Normal
Na_2-EDTA	:	Sodium salt of ferric ethylene diamine tetraacetate
NAA	:	a-Napthelene acetic acid
NaC1	:	Sodium chloride
NaOH	:	Sodium hydroxide
No.	:	Number
NS	:	Non-significant
pH	:	Negative logarithm of hydrogen ion concentration (-log [H])
UK	:	United Kingdom
USDA	:	United States Department of Agriculture
UV	:	Ultra violet
var.	:	Variety
µg	:	Microgram

ACKNOWLEDGEMENT

All praises gratitude are due to the Almighty Allah the great, the gracious, merciful and supreme ruler of the universe to complete the research work and thesis successfully for the degree of Master 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, Genetic Resource and Seed Division, 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 deems it a proud privilege to acknowledge her gratefulness, boundless gratitude and the best regards to her respectable Co-supervisor Professor Dr. Md. Sarowar Hossain, Department of Genetics and 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 professor Abu Akbar Mia , 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.

Special appreciation and warmest gratitude are extended to Professor Dr. Md. Shahidur Rashid Bhuiyan, and Assistant professor Firoz Mahmud, 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 research work. His contribution, love and affection would persist in the memory of the researcher for countless days.

The author humbly desires to acknowledge his heartiest appreciation and cordial thanks to Chadian Kumar Saha, Principal Scientific Officer, Md. Golam Mostafa, Senior Scientific officer, BJRI, Dhaka, who helped so much in conducting the research work.

The author feels much pleasure to convey the profound thanks to her friends Hasina Begum, Tazul Islam, Shain Akter, Mahmudur Rahman,Shamsunnahar, Naznen Akter, and other well wishers for their co-operation, cheerfulness and help during the research. He is particularly thankful to Md. Abul Hasem and Kazi Shafiqul islam who encourage him to undertake and complete this research and thesis work.

Last but not the least the author expresses her deepest sense of gratitude and thanks to her beloved Mother, younger brother Mahid,Fahim and younger sister Popy and all the relatives for their blessings, enduring sacrifice, inspiration, co-operation, forbearance and endless love throughout her life to complete her higher study at Sher-e-Bangla Agricultural University, Dhaka.

Dated : July,2007
SAU, Dhaka

The Author

LIST OF CONTENTS

Contents (Contd.)

Contents (Contd.)

LIST OF TABLES

LIST OF PLATES

LIST OF APPENDICES

PLANT REGENERATION PROTOCOL OF KENAF (*Hibiscus canabinus* L.)

BY

AMENA SIDDIKA

ABSTRACT

An experiment was conducted during the period of July 2006 to May 2007 in the Genetic Engineering Laboratory, Genetic Resources and Seed Division, Bangladesh Jute Research Institute, Dhaka, Bangladesh.. Kenaf variety HC-2 was used to investigate the *in vitro* regeneration potentiality. Different concentrations and combinations of hormones, vitamins, $FeSO_4$ and surfactant (F-68) were supplemented to MS medium to observe callus induction and regeneration ability of kenaf. In cotton-supported MS liquid medium, the germination percentage was higher (91.00%) compared to agar supported MS medium (82.34%). Eight days old seedlings were highly responsive to shoot regeneration. Among the phytohormone combination BAP (3.0 mg/l) and IAA(0.5 mg/l) with MS supplemented showed the highest shoot regeneration. In different concentrations of vitamin, the highest percentage of shoot regeneration was obtained (81.25%) at 4.0 mg/l vitamin concentration. Among different concentrations of surfactant, the highest percentage was observed (81.00%) in 0.5% surfactant concentration. Using different concentration of $FeSO_4$, the highest percentage of shoot regeneration was (91.67%) at 2.0 mg/l $FeSO_4$ concentration. In present study, an efficient protocol for the plant regeneration has been developed which could be further used for genestic transformation in kenaf.

CHAPTER I
INTRODUCTION

INTRODUCTION

Kenaf *(Hibiscus cannabinus)* is a fibre producing plant . .It is called "roselle" in India and in Thailand as "Saimi jute". However, it is known as kenaf in Bangladesh. Like Jute and mesta, kenaf has got economic value for fibre yield and biomass production. It is a world wide adapted potential fibre producing crop. Kenaf is a crop of tropical african origin . Now a days kenaf is widely distrubeted in the tropics and subtropics region. It is domesticad for its edible seeds ,subsequently for its leaves, young shoots and flower part as well (Maiti, 1997). It required monthly temperatures of 25-30^{0}C, a rainfall of 140-270 mm per month and high air humidity (> 70%). *H. cannabinus* has a long vegetative period (180-237 days), when sown starting from March 15 to mid- May in India.

H. cannabinus belong to the Malvaceae family. It is an allotetraploid Plant (2n =4x=72) with *H. asper Hook. f.* as one of the likely parental species and perhaps H. mechowii Garcke as the others. Kenaf is usually propagated by seed but can also be grown from stem cutting. Cultivar improvement in kenaf follows breeding methods is commonly applied to self pollinating crops, such as line and pedigree selection after intervarital crossing and back crossing. 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, 193.00 thousand tons of kenaf were produced in 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, Australia and USA (Ahmed an Vossen, 2003). The crop *Hibiscus cannabinius*, was selected by the United States Department of Agriculture from 500 crop species as the most promising non wood fibre alternative for pulp and paper

production. Through the next two decades, an extensive research programme was undertaken in the USDA for field production and the paper making characteristics of kenaf. One of the major constrains to increase kenaf productivity is the non availability of modern varieties, as well as infection by fungi, bacteria, virus, and nematodes. To improve the agronomic characters of kenaf, conventional breeding methods were practiced. The limitations of conventional breeding include narrow genetic base of the cultivated species, the length of time needed for successfully depelping crops cultivers, the difficulty in breaking gene linkages between useful and useless traits etc. In order to produce desirable lines of kenaf with high fibre yield and higher biomass production an alternate technique is necessary.

Modern biotechnological techniques may also be utilized conveniently to overcome incompatibility barrier trough fusion of protoplasts from vegetative cells of interspecific, inter generic and interfamilial group. However, in several instances callus derived from fused protoplasts could not be induced to regenerated plantlets (Rao 1885) Biotechnology is a recently developed novel approach, which included a range of techniques. Together these techniques comprise a powerful force to produce modified biological products according to specific objectives. Remarkable success have bee demonstrated for the improvement of numerous economic and food crops during the last twenty years using these techniques particularly in those for which conventional crop breeding has been less effective.

Plant tissue culture, a branch of biotechnology offers an efficient method for rapid propagation, production of pathogen free material and germplasm preservation. Tissue culture of a crop is also a prerequisite for the improvement of a crop through genetic transformation/*in vitro* regenerated plants in some cases give rise to spontaneous variants induced genetic variation. These

somaclonal variants in some cases can be useful for the selection of desirable genetic variants. The exploitation of heritable somaclonal variants has been used in various plant improvment strategies (Larking and Scowcroft, 1981; Evans, 1989; Larkin *et al,* 1989 and Philips *et al,* 1994). Recently, plant regeneration has already been reported from the explants of kenaf, which assures the exploitation of the species through tissue culture.

For improvement of a species through genetic engineering need appropriate regeneration protocol 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. However, callus induction and plant regeneration from explants require the presence of appropriate combination and concentration of hormone in the culture media (Ehsanpour and JHones, 2000)

The regeneration ability depends on optimum growth conditions, suitable explants and varieties. In the present investigation, attempts were made to establish a suitable *in vitro* regeneration protocol for kenaf under the following objectives:

1 .To develop a suitable regeneration protocol for kenaf.

2. To study the effcet of different phytohormone on plant regeneration in kenaf.

3. To study the effect of $FeSO_4$, Vitamins and Surfactants on plantlet regeneration in kenaf.

CHAPTER II
REVIEW OF LITERATURE

REVIEW OF LITERATURE

Kenaf (*Hibiscus cannabinus*) is one of the most fibre and paper pulp yielding and biomass producing crop in the world. The constrains of cultivation of this crop are insect infestation, pests, diseases and low yield potentiality. Pest and insects hampers the production of kenaf cultivars in Bangladesh. To overcome this problem, traditional breeding is employing in different countries including Bangladesh. However, traditional breeding is time consuming and has a risk of assimilation of undesired genes. In contrast, plant tissue culture technique allows the rapid regeneration of pathogen free plantlets and genetic engineering offers a direct method of plant breeding that selectively introduces new gene or genes into the plant genom. However related literature regarding cell culture are given under different sub heading.

2.1 Concept of tissue culture

Plant tissue culture or the aseptic culture of cells, tissues and organs, is an important tool in both basic and applied studies. Haberlandt, a German plant physilologist, introduced the concept of totipotency, that all living cells containing a normal complement of chromosomes should be capable of regenerating the entire plant. Regeneration from different explants eg. leaf stem, cotyledon, hypocotyle on defined nutrient medium 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 is called callus. The medium can be subsequently manipulated to obtain root, shoot and embryos, which then develop into whole plants from this undifferentiated callus. This process in known as plant regeneration. Conventional breeding techniques are lengthy processes and take long time for crop improvement. The techniques of plant tissue culture have been developed as new and powerful tool for crop improvement (Carlson, 1975; Razdan and

Cocking 1981) and received wide attention of scientific world (Amato, 1978; Skirvin, 1978; Larkin and Scowcroft, 1981). There are many crop species, which was successfully regenerated through plant cell culture during the last decades . For many corps like tobacco, rice and some other horticultural crops tissue culture technique already been developed.

2.2 Tissue culture of kenaf (*Hibischus cannabinus)*

In vitro regeneration has been quite difficult among the species of kenaf, mesta and jute. It appears that kenaf and jute have notorious recalcitrant tissue and regeneration is sporadic. Regeneration has only been reported from meristemetic tissue, other than totally differentiated tissue, like callus. In kenaf, regeneration was observed from cotyledons with attached petioles and hypocotyls where meristematic tissue was present. As part of the kenaf improvement programme at Mississippi State University, Larkin and Scowcroft (1981) employed tissue culture as a means for introduction of new or altered traits into kenaf. Regenerates often display altered phenotypes, termed somaclonal variation. The exploitation of heritable somaclonal variants has been used in various plant improvement strategies Larkin and Scowcroft (1981); Evans (1989) ; Larkin *et al.* (1989); Phillips *et al.* (1994). However, success has been sporadic. Reichert and Liu, (1994) has optimized adventitious shoot regeneration protocols for kenaf, starting with internodal stem and leaf sections. They regenerated from three new varieties: Everglades 41 (E41), Guatemala 45 (G45), and G48 by utilizing *in vitro* culture technique.

A callus is an amorphous mass of loosely arranged thin-walled parenchymatous cells arising from the proliferating cells of parent tissue (Dodds and Roberts, 1990). Different explants and different combinations of growth regulators have an influence on callus induction from various *Hibischus cannabinus* varieties. Various explants like cotyledons with attached petiole, hypocotyle containing

meristemetic zone and shoot apexes were used for regeneration of kenaf. Mc Lean *et al.* (1992) first reported kenaf regeneration. They used internodal stem as explants supplemented with different combinations and concentrations of auxins and cytokinins (PGR's).

A direct and simple regeneration procedure using shoot apex was reported by Zapata *et. al.* (1999). Regeneration from internodal section was reported by Reichert and Liu (1994). They took internodal stem sections from field-grown plants of ' Everglades 41' ('E41'), surface-disinfested, 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/1, thidiazuron (TDZ) with pH 5.8 for shoot initiation. Purwati and Sudarmadji (1998) studied the response of five kenaf accessions for shoot regeneration and established regeneration protocol for kenaf. From cotyledons with attached plumules Purwati and Sudarmadji (1999) used MS based medium containing BAP (2 mg/l) and GA_3(0.5mg/l) for callus induction. Calli produced in this culture were than transferred into MS- based medium containing BAP (2mg/l) and GA_3(5mg/l) for shoot initiation.

Cotton (*Gossypium hirsutum* L.) and kenaf (*Hibiscus cannabinus* L.) belong to the *M*alvaceae family, and both are used as sources of fibres. Zapata *et al.*(1999) worked on cotton and kenaf. They used shoot apex as explant. The shoot apices of both crops multiplied successfully without intervening callus formation, and no significant differences among cultivars were found. An average of 58% of the cotton shoot apices initiated shoots and rooted in full strength Murashige and Skoog (1962) plus vitamins. For kenaf, an average of 92% of shoot apices initiated shoot and rooted in full strength Murashige and Skoog plus vitamins and 0.1 mg/l BA .

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 cannbinus* L. var) (HC-2) on MS medium supplemented by different levels of IAA or NAA and BAP .

Herath *et al.* (2004) reported that the number of shoots regenerated varied with the cultuvar, explant, and the BA concentration.The highest number of shoots (11/explant) was developed in cv Tainung 2 (T 2) shoot tips cultured in MS medium supplemented with 2 mg /l BA. Srivatanakul *et.al.*, (2000) developed a medium that stimulates multiple shoot initiation from explants of kenaf (*Hibiscus cannabinus*). Adventitious shoot formation on a shoot induction media supplemented with combinations of 2,4-D (0, 0.5, 2.3 umol/1) and thidiazuron (0,1,5,20, umol/1) was evaluated. Multiple shoot induction medium with 1 umol TDZ /1, resulted the highest number of regenerated shoot per explants

Tewari *et al.* (1999) reported that MS medium supplemented with 2, 4-D induced callus in 100% explants of jute. Khatun (2001) cultured *in vitro* grown cotyledons (with attached petioles) of *C. capsularis* in agar solidified MS medium supplemented with 0.5 mg/l IAA and different concentration of BAP (2, 3, 4 or 5 mg/l) and noticed poor performance in cullus induction and shoot regeneration.

2.3 Influence of surfactants on plant regeneration

Khatun *et al*(1992) conducted an experiment of stimulation of differentiation in jute cotyledon cultured with pluronic F-68. They reported that the addition to MS-based medium of 0.1 or 0.5% (w/v) of either commercial grade pluronic F-68 or a purified fraction obtained by passage through silica gel, stimulated shoot production from the petioles of cotyledons of vars. D154 and C134 . The

implications of these results are discussed in relation to the potential value of non-ionic surfactants as additives to plant culture media for stimulating growth and differentiation.

Lowe *et al.* (1993) showed that a novel approach to the growth of cultured plant cells, tissues and organs by supplementation of culture media with low concentrations (<1.0% w/v) of surfactants. They use the plant, *Hibiscus cannabinus*, *Solanum dulcamara* and *Corchorus capsularis* demonstrated and that the considerable growth stimulation effects of pluronic (poloxamer) co-polymers in both liquid and semi-solid systems. Kumar *et al.* (1991) reported that the non-ionic, copolymer surfactant , pluronic F-68 is a valuable growth-promoting supplement in plant culture systems. For example, addition of low concentrations of Pluronic F-68 to culture media stimulated growth of callus, isolated protoplants and *Agrobacterium rhizogens* transformed roots of *Solanum duleamara.*

CHAPTER III
MATERIALS AND METHODS

MATERIALS AND METHODS

The present investigation was carried out in the Genetic Engineering Laboratory of the Genetic Resource and Seed Division, Bangladesh Jute Research Institute from July 2006 to June 2007.

3.1 Experimental materials The variety (HC-2) of the plant kenaf used as experimental material .

3.1.1 Explant

Cotyledons with attached petioles of kenaf (var.HC-2) were used as explant.

3.1.2 Media used

MS medium supplemented with different phytohormone and surfactants.

3.1.2.1 Medium used for seed germination

MS (Murasighe and Skoog, 1962) basal medium was used as a source of salts and vitamins. Clinical cotton-supported MS liquid medium was used for seed germination. Agar- based MS solidified medium was also used for seed germination.

3.1.2.2 Medium used for shoot regeneration

Agar based MS solifified medium supplemented with IAA (0.5 mg/l) and BAP (0.0,1.0, 2.0, 3.0, 4.0, 5.0mg/l) was used for plant regeneration. MS medium without hormone was used as a control.

3.2 Media preparation

3.2.1 Preparation of stock solutions

The preparation of different stock solutions for macronutrients, micronutrients, Iron, vitamins, hormones were done precisely according recommendate dose. Chemical composition of macro and micronutrients, iron, vitamin and hormones are given in Appendix.I.

i) Stock solution A (Macronutrients)

The stock solutions for 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 weighted accurately and dissolved in 750 ml of distilled water and final volume was made up to 1000 ml by further addition of distilled water. The stock solution was stored in a refrigerator at 4^0 C for later use.

ii) Stock solution B (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. The stock solution was labeled and stored in a refrigerator at 4^0C for further use.

iii) Iron source C

The required amount of $FeSo_4$ was weighted and added during the preparation of medium.

iv) Stock solution D (vitamins)

Each of the desired ingredients except myo-inositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots stored at 20^0 C. Myo- inositaol was used directly at the time of media preparation.

v) Stock solution for hormones

The hormones, IAA and BAP (hormone supplements) were used in the present investigation. For the preparation of stock solution of any of these hormones, 10 mg of each of the hormone power was taken in a clean beaker and dissolved in 1.0 ml of the particular solvent. IAA was dissolved in few drops of absolute alcohol and BAP was dissolved in 0.1N NaOH. The mixtures were then taken in a 100 ml volumetric flask and volume was made up to 100 ml by the further addition of distilled water. The solution was then stored at 4^0C.

3.2.1.1 Steps followed for the preparation of culture media.

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

3.2.1.2Preparation of MS medium

To prepare 1 liter (1000 ml) of MS medium, the following steps were followed:

i) One hundred ml of macronutrients, 10 ml of micronutrients, 0.028 gm $FeSo_4$ and 10 ml of vitamins were taken from each of these stock solutions into a 2 liter Erlenmeyer flask on a magnetic stirrer.

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

iii) Thirty gram of sucrose was added to this solution and agitated gently to dissolve completely.

iv) 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.

v) pH of the medium was adjusted to 5.8 with a digital pH meter by adding NaoH or HCI (1% solution) whichever was necessary.

vi) The whole mixture was then made up to 500 ml with further addition of distilled water.

vii) Agar was dissolved in 500 ml distilled water and hot liquid agar was added with 500 ml medium and mixed thoroughly.

viii) 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 helps of a permanent marker to indicate specific hormone combinations.

3.3 Sterilization techniques

3.3.1 Sterilization of culture media

All prepared media were autoclaved at 15 psi pressure and 121^0 C temperature for 20 min. All autoclaved media were marked with permanent marker to indicate specific hormone combinations.

3.3.2 Sterilization of glassware's and instruments

Flasks, pipettes, small instrument like forceps, scalpels, micropitette, were prepared for autoclaving by wrapping with brown papers. Tap water was autoclaved in 500 ml conical flasks. Vials were capped with plastic caps and then were sterilized in an autoclave at a temperature of 121^{0}C for 20 minutes at 15 psi pressure.

3.3.3 Sterilization of culture room and transfer area

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

3.3.4 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 and forceps were

immersed into 95% alcohol contained in a test inside the cabinet. Both of the hands were rinsed with 95% alcohol. All measures were taken to obtain maximum contamination free condition during the working period.

3.4 Culture techniques

The following culture techniques were employed in the present investigation:

i) Raising of *in vitro* seedling

ii) Cotyledon culture

iii) Multiplication of shoot from shoot apex

iv) Influence of different concentration of vitamins, iron and surfactant for plant regeneration in kenaf

3.4.1 Raising of *in vitro* seedling

Healthy seedling production was found to be one of the major criteria for plant regeneration from *Hibiscus cannbinus* explants and thereby to be successful in genetic transformation. Seeds of *H. cannabinus* (var.HC-2) were surface sterilized by immersing in absolute alcohol for 1 min. and then in 0.1% Mercuric Chloride for 20 min. Seeds were thoroughly washed with autoclaved water for 4-5 times. The sterlized seeds were transferred on clinical cotton supported MS (Murashige and skoog, 1962) liquid medium and agar (Sigma, UK, 0.8%, w/v) based MS solidified medium in 500 ml conical flasks. Each flask contained 12-15 seeds and was placed in a growth room with 27 ± 2^{0}C temperature under 1500 lux fluorescent illumination with 12h photo period.

3.4.2 Cotyledon culture

Cotyledons with attached petioles were taken from *in vitro* raised *H. cannabinus* (HC-2) seedlings. Seedlings were used for cotyledon culture after the emergence of the shoots between the cotyledons. Precaution was taken for emerging shoots were not remained attached with the petioles. Four cotyledons with attached petioles were cultured on MS medium in 250 conical flasks and gently pressed on the surface of the sterilized culture medium so that the cut end of the petioles were immersed into the medium to a depth of 2 mm. Various combinations and concentrations of hormone IAA (0.0, 0.5 and 1.0 mg/1) and BAP (0.0, 1.0, 2.0, 3.0,4.0, and 5.0 mg/1) were used . The cultures were maintained in a growth room with 27 ± 2^0C temperature under 1500 lux fluorescent illumination of 12 h photperiod. The conical flasks were checked daily to note the response and the development of contamination. Number of shoots regenerated from the cotyledons in the culture medium was recorded.

3.4.3 Multiplication of shoot from shoot apex

Shoot apices were taken from *in vitro* raised *H. Cannbinus* seedlings. Four shoot apices were placed on MS medium in 250 ml conical flasks. Various combinations and concentrations of hormone IAA (0.5 and 1.0 mg/1) and BAP (1.0, 2.0 and 4.0 mg/1) were used. The cultures were maintained in a growth room with 27 ± 2^0C temperature under 1500 lux fluorescent illumination of 12 h photoperiod. The conical flasks were checked daily to note the response and also for the checking of contamination. Number of shoots multiplied from shoot apices of *H. cannabinus* in the culture medium was recorded.

3.4.4 Influence of different concentration of vitamins on plant regeneration

The following culture techniques were employed in the present study :

a) Axenic culture

b) Explants culture

a) Axenic culture

Sterilized seeds were placed into sterilized seed germination medium in culture vessels. Twelve seeds were placed in each flask. The culture was then incubated in dark till the germination of seeds. These were then transferred to 12 hours light for normal seedling growth. Seven to Eight days old seedlings were used as source of contamination-free explants.

b) Explants culture

The seedlings raised in axenic culture and explants were used as the source of explants. Twelve explants were placed in MS media three replications of containing different concentration of vitamin supplemented with 3.0 mg/l BAP and 0.5 mg/1, IAA at 0.0, x1 times (2.0mg/1) x2 times (4.0 mg/1), x3 times (8.0mg/1) x4 times (16.0mg/1). Cultured explants were placed under fluorescent light in a growth room with controlled temperature (27+-2^0 C) The flasks were checked daily to note the appearance of callus and shoot regeneration .

3.4.5 Influence of surfactants (pluronic F-68) on plant regeneration

The following culture techniques were employed in the present study:

a) Axenic culture
b) Explants culture

a) Axenic culture

Discussed in axenic culture above

b) Explants culture

The seedlings raised in axenic culture and explants culture were used as the source of explants. Twelve explants were inoculated in three replication of containing different concentration of pluronic F-68 (0.0, 0.001, 0.01, 0.1, 0.5)% supplemented with 3.0 mg/l and 0.5 mg/l IAA. In three replication containing explants were placed under fluorescent light in a growth room with controlled temperature (27±2°C). The flasks were checked daily to note the appearance of callus and shoot regeneration.

3.4.6. Influence of $FeSO_4$ on plant regeneration from the cotyledons of *H.cannabinus*

The following culture techniques were employed in the present study:

a) Axenic culture
b) Explants culture

a) Axenic culture

Discussed in axenic culture above

b) Explants culture

The seedlings raised in axenic culture and explants culture were used as the source of explants. Twelve explants were placed in three replications containing different concentration of $FeSO_4$ supplemented with 3.0 mg/l BAP and 0.5mg/l IAA. 0.0(control), x 1 times(1.0mg/l) x2 times(2.0 mg/l) x3 times(3.0mg/l)x 4 times(4.0mg/l). Cultured explants were placed under fluorescent light in a growth room with controlled temperature (27±2°C).

3.5 Data recording

To investigate the effects of different treatments and responses of different varieties to callus induction and plantlet regeneration, data were collected on the basis of following parameters.

3.5.1 Seed germination

The number of germinated seeds was recorded. The percentages of germinated seeds were calculated on the basis of the number of seeds placed and the number of seeds germinated.

$$\text{Percent seed germination} = \frac{No.of\ total\ seeds\ ger\min ate}{No.of\ seeds\ placed} \times 100$$

3.5.2 Callus induction

3.5.2.1 Generally callus initiation started after 9-10 days of incubation of explants. The number of callus initiated over a number at different period was recorded.

Number of calli / conical flask (Per cent callus induction)

The number of explants producing callus in each petridish 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.

$$\text{Percent callus induction} = \frac{No.of\ explants\ induced\ calli}{No.of\ explant\ incubated} \times 100$$

3.5.3 Plant regeneration

3.5.3.1 Days to regeneration

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

3.5.3.2 Number of shoot/ callus

The number of shoot producing plantlets in each Petri dish was recorded. The percentage of shoot induction was calculated on the basis of the number of

Explants placed and the total number of callus induced.

$$\text{Per cent plant regeneration} = \frac{No.of\ calli\ with\ shoots}{No.of\ inoculated\ calli} \times 100$$

3.6 Statistical analysis

The data for the parameters under present experiments were statistically analyzed wherever applicable. The analyses of variances for different parameters were performed and means were compared by the Duncan's Multiple Range Test (DMRT).

CHAPTER IV
RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

in vitro plant regeneration of kenaf variety (HC-2) was conducted using various hormone concentrations. The effect of FeSO4, vitamin and surfactants was also observed for plant regeneration. The results along with the discussion are mention below.

4.1 Percent seed germination

Percent seed germination from kenaf variety was found to be higher on cotton supported MS liquid medium (91.00%) compared to agar based MS solidified liquid medium(82.34%). This finding is similar to the finding of Khatun (2001) who reported that germination percentage of jute (*C.capsularis)* was found higher in cotton-supported MS liquid medium than the agar based MS solidified medium (Table-1)

Table- 1. Germination of kenaf Seeds on clinical cotton supported MS liquid medium and agar based MS solidified medium

Type of media	Total number of seed	Number of germinated seeds	Percent seed germination (%)
Cotton supported MS liquid medium	50.00	46.00a	91.00a
Agar based MS solidified medium	50.00	40.69b	82.34b

Figure followed same letter in column do not differ significant by DMRT.

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

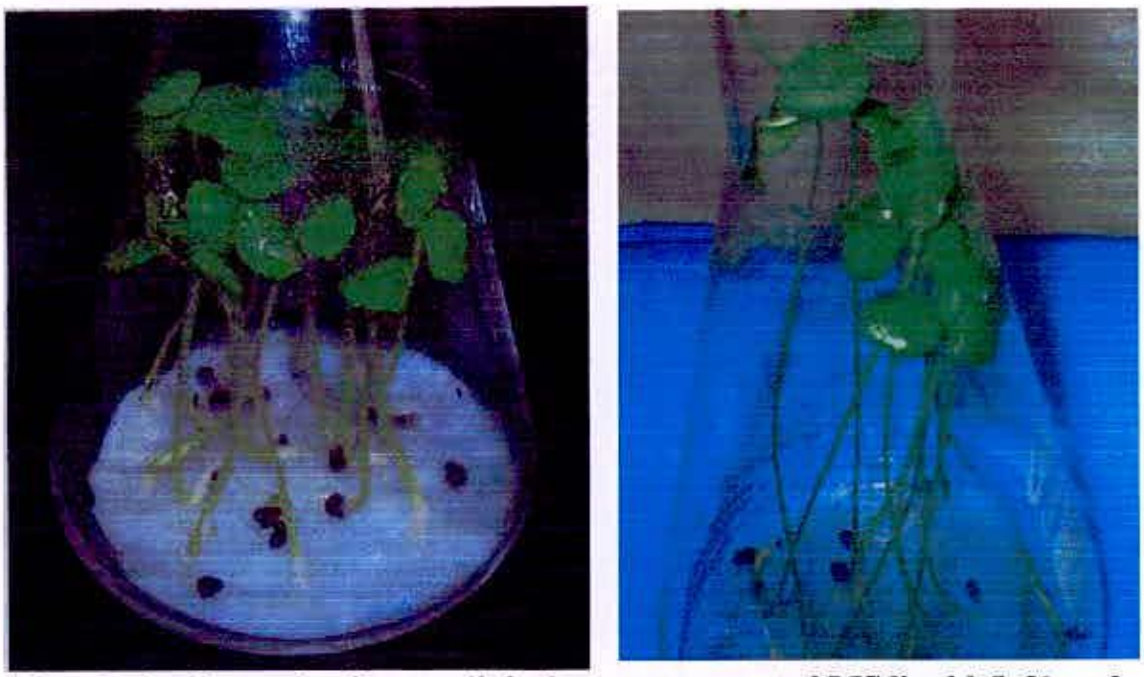

Plate-2: Seed germination on clinical cotton-supported MS liquid (left) and agar based MS solidified medium (right)

4.2 Optimization of plant regeneration from the explants of *H. Cannabinus* with different concentrations of BAP

Different concentration of BAP and IAA (0.5 mg/l) were used for callus induction and shoot regeneration using cotyledonary petioles as explants of kenaf var. HC-2.

4.2.1 Percent callus induction

Percent callus induction of kenaf in IAA (0.5 mg/l) with different concentrations of BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0 mg/l) was presented in Table 2. The highest callus induction percent (76.68 %) was observed in IAA (0.5 mg/l) with BAP (3.0 mg/l) concentration and the lowest percentage of callus induction was (48.33%) observed in IAA (0.5 mg/l)with (1.0 mg/l) Callus induction was not occurred in control treatment i.e. without BAP .(Table 2)

4.2.2 Number of shoots per callus

The highest number of shoots per callus was obtained (3.60) at BAP (3.0mg/l) with IAA (0.5 mg/l). The lowest number of shoots per callus obtained (1.60) in MS supplemented with BAP (0.0mg/l) and IAA (0.5mg/l) (Table 2). Number of shoot per callus without hormone was zero. This observation is similar to the finding of Haque, M. (2005) who reported that the highest number of regenerated shoots were obtained from cotyledons with attached petioles explants of *H. cannabinus* on MS medium supplemented by IAA (0.5mg/l) and BAP (3. 0mg/l)

Table 2- Effect of BAP on callus induction and shoot formation.

IAA (mg/l)	BAP (mg/l)	Calli/flask*	Percent callus induction (%)	Number of shoot /callus
0.5	0.0	0.00e	0.00e cd	0.00d
	1.0	3.20cd	48.33d	1.60bc
	2.0	3.00cd	50.00a	2.00b
	3.0	4.60a	76.68bc	3.60a
	4.0	3.60bc	61.00bc	1.80bc
	5.0	3.40bc	56.67e	1.70bc

Figure followed same letter in column do not differ significant by DMRT.

* Each flask contains 4 cotyledones.

Plate-3: Callus induction from cotyledons with attached petioles on MS medium supplemented by IAA (0.5mg/l) and BAP (1.0 mg/l)

Plate-4: Callus induction from cotyledons with attached petioles on MS medium supplemented by IAA (0.5mg/l) and BAP (3.0 mg/l)

4.3 Effect of duration on plant regeneration .

4.3.1 Percent Shoot regeneration

In vitro plant regeneration depends on a number of factors including proper concentration of growth regulators used and the response of varieties of plant material. Cotyledons with attached petioles of kenaf verities HC-2 was incubated on MS media supplemented with BAP (3.0 mg/L) and IAA (0.5 mg/L) for shoot regeneration. The highest percent shoot regeneration was (81.25%) which was observed in 8 days old explants and the lowest percent shoot regeneration is (43.75%) which was observed in 10 days explants.(Table-3) This result correlated with the findings of Haque, M (2005) who reported that 7-8 days old seedling showed the highest percentage of shoot regeneration.

4.3.2 Number of shoot per cotyledon

The highest number of shoot per cotyledon at BAP (3.0 mg/l) and IAA (0.5 mg/l) was obtained (1.96) 8 days old explants. The lowest no. of shoot per cotyledon was obtained (1.27) in 10 days old explants.

Table 3: Effect of duration plant regeneration .

Treatment (age of explants)	Number of explants showing shoot	Percent shoot regeneration (%)	Number of shoot per cotyledon
7days	2.75ab	68.75ab	1.34b
8days	3.25a	81.25a	1.96a
9days	2.00ab	50.00ab	1.29b
10days	1.75b	43.75b	1.27b
CV %	36.34	36.34	25.52
LSD	1.417	35.42	0.597

Figure followed same letter in column do not differ significant by DMRT.

* Each flask contains 4 cotyledones.

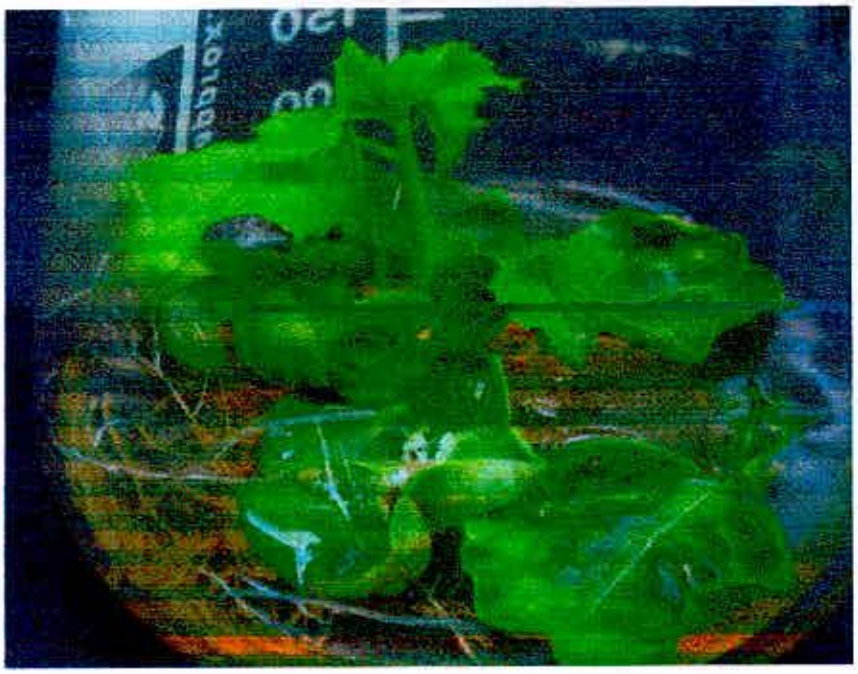

Plate-5 Shoot regeneration in kenaf variety (HC-2) on 8 days old explants

Plate-6 Shoot regeneration in kenaf variety (HC-2) on 10 days old explants

4.4 Effect of different concentration of vitamins on plant regeneration

4.4.1 Effect of different concentration of vitamins

Mean value due to different concentration of vitamins for number of explants showing shoot, percent shoot produced per cotyledon were significant, indicating the effect in variation among the vitamin concentration used for study, X2 times (4.0 gm/l) (twice than the normal vitamin concentration in MS media) concentration was found to be the best. Maximum number of explants showing shoot was (3.25) and the highest percent of shoot regeneration was (81.25%) found t this concentration. Number of explants showing shoot and percent shoot regeneration gradually decreased with the increased of vitamin concentration (Table-4).

4.4.2 Number of shoot/cotyledon:

The highest number of shoot per cotyledon was (1.50) at X2 times(4.0mg/l) vitamin concentration and the lowest number of shoot per cotyledon was obtained (1.21) at control(0.0mg/l) and X4 times(16.0mg/l) vitamin concentration. This finding is similar to the finding of Khatun (2001) who found the best performance in shoot regeneration on the combination of MS media with X2 times vitamin concentration. No shoot regeneration ability was found without vitamin. It might be concluded that MS media with X2 times vitamins combination was favorable for higher percentage of shoot regeneration (Plate No. 7 & 8).

Table-4: Effect of different concentration of vitamins on plant regeneration of kenaf variety.

Vitamin Concentration (mg/L)	Number of explants showing shoot	Percent shoot regeneration (%)	No. of shoot per cotyledon
Control (0)	1.50c	37.50b	1.50ab
X1 times (2.0)	2.50c	62.50ab	1.38b
X2 times (4.0)	3.25c	81.25a	2.00a
X3 times (8.0)	2.25bc	56.25ab	1.30b
X4 times (16.0)	1.76bc	43.75b	1.21b
CV %	24.00	37.41	23.74
LSD	0.84	32.42	0.539

Figure followed same letter in column do not differ significant by DMRT.

Each flask contains 4 cotyledones.

Plate-7 Shoot regeneration in kenaf variety (HC-2) on x2 times (4.0 mg/l) vitamin concentration

Plate-8 Shoot regeneration in kenaf variety (HC-2) on x4 (16.0 mg/l) times vitamin concentrations

4.5 Effect of different concentration of $FeSO_4$ on plant regeneration

4.5.1 Effect of $FeSO_4$ concentration:

Mean value due to different concentration of $FeSO_4$ for number of explants showings shoot, percent shoot regeneration and number of shoot produced per cotyledon were significant, indicating the effect in variation among the $FeSO_4$ concentration used for the study, $FeSO_4$ concentration X2 times (2.0 mg/l) was found to be the best. The highest percent shoot regeneration was (91.67%) found at this concentration. Percent shoot regeneration gradually decreased with the decreased of $FeSO_4$ concentration (Table-5).

4.5.2 Number of shoot / cotyledon:

The highest no. of shoot per cotyledon was (1.84) at X2 times(2.0mg/l) $FeSO_4$ concentration and the lowest no of shoot per cotyledon was obtained (0.17) at control (0.0 mg/l) $FeSO_4$ concentration. This finding was similar to the findings of Khatun (1996) who found best performance in shoot regeneration on the combination of MS media with x2 times (twice than normal concentrations) $FeSO_4$ concentration. Number shoot regeneration ability was found without $FeSO_4$. It might be concluded that MS media with x2 times $FeSO_4$ combination was favorable for higher percentage of shoot regeneration (Plate No. 9 & 10)

Table-5: Effect of different concentration of $FeSO_4$ on plant regeneration of kenaf variety

FeSO4 concentration (mg/L)	Number of explants showing shoot	Percent shoot regeneration (%)	Number of Shoot / cotyledon
Control (0)	1.34c	33.33c	1.17c
X1 times (1.0)	2.00bc	50.00bc	1.62ab
X2 times (2.0)	3.67a	91.67a	1.84a
X3 times (3.0)	3.00ab	75.00ab	1.36bc
X4 times (4.0)	2.67abc	66.67abc	1.64ab
CV %	28.83	28.84	13.14
LSD	1.375	34.38	0.377

Figure followed same letter in column do not differ significant by DMRT.

* Each flask contains 4 cotyledones.

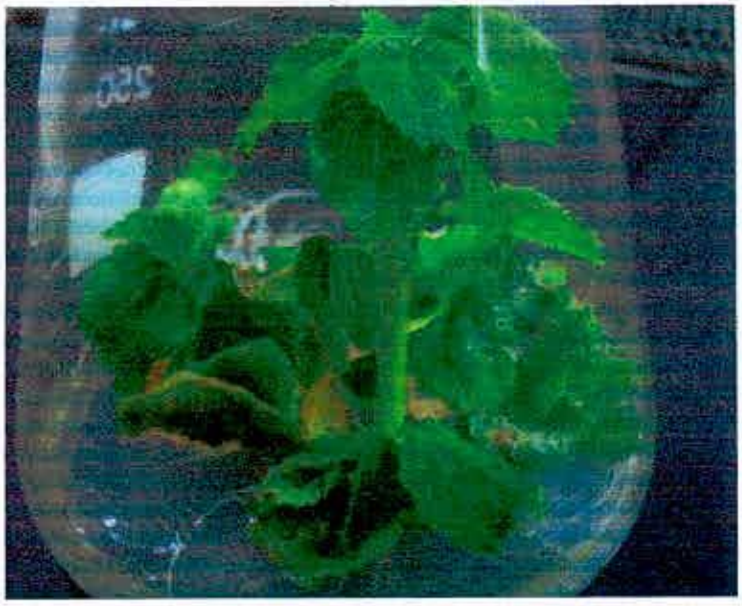

Plate-9 Shoot regeneration in kenaf variety (HC-2) on x2 times (2.0 mg/l) $FeSO_4$ concentration

Plate-10 Shoot regeneration in kenaf variety (HC-2) on x4 times (4.0 mg/l) $FeSO_4$ concentration

4.6 Effect of surfactant (Pluronic F-68) on plant regeneration from catyledons of kenaf variety

4.6.1 Effect of surfactant concentration:

Difference in number of shoot production from cotyledon and percentage of explants producing shoots have been observed in the presence of various concentration of surfactants are shown in Table-6. The highest percentage of cotyledons producing shoot was recorded (81.00%) in 0.5% surfactant concentration and the highest number of cotyledons producing shoots was obtained (11.22). The lowest percentage of cotyledons producing shoots was recorded (59.10) at control (0 mg/l) and the lowest obtained (4.26). However, it was observed shoots increased at the surfactant concentration increased. This findings is similar to the findings of Khatun (2003) who found best performance in shoot regeneration on the combination of MS media with 0.5% surfactant (Table-6).

Table-6: Effect of Different concentration of surfactant (F-68) on plant regeneration

Variety	Concentration of pluronic F-68 (%)	Percentage of cotyledons producing shoots (%)	Number of cotyledons produced shoots
HC-2	Control (0)	59.10c	4.267d
	0.001	67.83d	4.417d
	0.01	71.08c	7.390c
	0.1	76.08b	10.25b
	0.5	81.00a	11.22a
CV %		1.45	3.30
LSD		1.943	0.469

Figure followed same letter in column do not differ significant by DMRT.

* Each flask contains 4 cotyledones.

CHAPTER V
SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

In the experiment, investigation was carried out to study the seed germination, days to plant regeneration, effect of different concentration of hormones, vitamins, $FeSO_4$ and surfactants on plant regeneration in kenaf plant using cotyledons with attached petioles as explants. In the present study, it was observed that cotton supported MS liquid medium performed better than agar based MS solidified medium for seed germination.

Cotyledons (with attached petioles) of kenaf were cultured on MS medium supplemented by various BAP concentration to observe the callus induction performance of cotyledons. For the study, different hormone concentrations showed different regeneration performance. The highest percentage of callus indication (76.68%) was found of MS medium + IAA (0.5 mg/l) with BAP (3.0 mg/l). No regeneration was obtained on MS medium without hormone (MSO). The highest percentage of shoot regeneration (82.34%) was found in 8 days old seedlings and the lowest percentage of shoot regeneration (43.75%) was found in 10 days old seedlings.

Different concentration of vitamins were used to observe the efficient plant regeneration of kenaf. The highest percentage of shoot (81.25%) at X2 (4.0 mg/l) times and number of shoot per cotyledon (2.00). The lowest percentage of shoot regeneration was obtained (37.50%) at control (0.0 mg/l) vitamin concentration. Different concentration of $FeSO_4$ was used to observe the efficient plant regeneration. The highest percentage of shoot regeneration (91.67%) at X2 (2.0 mg/l) times and the lowest percentage of shoot regeneration was (33.33%) at control (0.0 mg/l) the maximum number of shoot per cotyledon was obtained (1.84) at x2 times $FeSO_4$ concentration. Difference

concentration of surfactant were used to observe the efficient plant regeneration. Maximum number of shoot per cotyledon (11.22) at 0.5% surfactant concentration and the highest percentage of cotyledons producing shoots was obtained (81.00%) at (0.5%) surfactant concentration.

In the present study, an efficient and reproducible protocol for the plant regeneration of kenaf *(Hibiscus cannabinus)* genotype has been developed using cotyledons with attached petioles. Genetic transformation depends on an efficient method of plant regeneration. This developed protocol could be used for genetic transformation in kenaf in future.

REFERENCES

REFERENCES

Ahmed, S. and Vossen, H.A.M.V. (2003). *Hibiscus sabdariffa.* In: Brink, M. and Escobin, R.P. (Eds.): Plant Resources of South-East Asia No. 17. Fibre Plants. Bachuys Publication, Leiden, the Netherlands. pp: 162-167

Amato. F. D. (1978). Chromosome number variation in cultured cells of regenerated plants In: T.A Thorpe (ed). Frontiers of Plant Tissue Culture. Canada. pp. l01-104.

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

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

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

Evans, D.A. (1989). Somaclonal variation: Genetic basis and breeding applications. Trends Genet. **5**: 46-50.

Herath, P.S., Suzuki, T. and Hattori. K .(2004). Multiple shoot regeneration of kenaf. Plant Cell Tissue and Organ Culture. **96**(6): 199-211.

Haque, M. (2005) Plant regeneration potentiality and *Agrobacterium* mediated genetic transfromation of two kenaf varieties (*Hibiscus cannabinus*) MS Thesis, Dept of Biotechnology, Agricultural University, Mymanshingh.

Kathun. A.. Naher Z.. Mahboob, S., Saha, C.K. and Bilkis, S. (2003). An Efficient Pritocol for Plant Regeneration from the Cotyledons of kenaf (*Hibiscus cannabinus* L.). Biotechnologv. **22**: 86-93.

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

Khatun, A., Laouar, A., Davey, M.R., Power, J.B., Mulligan, B.J. and Lowe, K.C. (1993). Effects of Pluronic F-68 on shoot regeneration from cultured jute cotyledons and on growth of transformed roots. Plant Cell, Tiss. and Organ Cult. **34:** 133-140.

Khatun, A. (1996). Effect of different levels of $FeSO_4$ shoot regeneration from the cotyledon of *C. capsularis.* Annual report. Bangladesh Jute Research Institute. Manik Mia Avenue, Dhaka pp-6-7.

Khatun, A., Davey, M.R., Laour L., Mulligan, B.J., Power, J.B. and Lowe, K.C. (1992). Stimulation of differentiation in Jute cotyledous cultured with pluronic F-68. plants tiss. cult. **2** (2): 75-80

Khatun, et.al (2003) Influence of Pluronic F-68 on Plants Regeneration from the explants of Kenaf (*Hibiscus Cannabinus L.)* B.J. Jute Fib. Res. Vol. **24**: 103-110.

Kumar, V. , Laour, L., Davey, M.R., Mellor, I.R., Mulligan, B.J and Lowe, K.C. (1991). Effects of Pluronic F-68 on callus growth and protoplast planting efficiency of *solanum dulcamara.* plant cell Rep. **10:**52-54

Lowe, et. al. (1993). Surfactant supplements systems in plant culture. Agro-food-Industry-Hi- Tech. 4(1):9-13.

Larkin, P.J., Banks, P.M.. Bhati, R.. Brettell. R.I.S., Davies, P.A., Ryan, S.A., Scowcroft, W.R., Spindler. L.H.. and Tanner, G.J. (1989). From somatic variation to variant plants: mechanisms and applications Genome. **31:** 705-711.

Larkin, P.J. and Scwcroft, W.R. (1981). Somaclonal Variation: A novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. **60:** 197-214.

Maiti, P.K. (1997). World Fiber Crops. Science Publication, Inc. New Hampshire, USA. pp: 41-61.

McLean, K.S., Lawrence, G.W. and Reichert, N.A. (1992). Callus induction and adventitious organogenesis of kenaf *(Hibiscus cannabinus* L.) Plant Cell Rep. **11:** 532-534.

Murashige: T. and e. F. (1962). A revised medium for rapid growth and bioass with tobacco. tissue cultures. Physiol. of Plant. **15**: 473-497.

Phillips. R.L., Kaeppler, S.M., and Olhoft, P. (1994). Genetic instability of plant tissue cultures: breakdown of normal controls. Proc. Nat. Acad. Sci. (USA). **91:** 5222-5226.

Purwati, R.D. and Sudarmadji, S. (1998). Shoot regeneration tissue culture of kenaf and jute. Proceed application of biotechnology in the improvement of jute, kenaf and allied fibers-phase i. IJO project. 23-25 November 1998, Beijing, China. pp. 56-65.

Purwati, R. D. and Sudarmadji, S. (1999). Response of five kenaf accessions to shoot regeneration. Journal of Penelitian Tanaman Industry. Indonesia. **5** (1): 31-37.

Reichert, N.A. and Liu, D. (1994). Protoplast culture and in vitro regeneration of kenaf. Conference Proc., Sixth Int. Kenaf Assoc. pp. 61-65.

Reichert, N.A. and Liu, D. (1994). Manipulations and regeneration of kenaf (*Hibiscus cannabinus* L.) in vitro. (Manuscript in preparation).

Rao, P.S. (1985). Plant protoplast: a new plant biotechnology. Current science. **54**(7):335-336.

Razdan, M.K. and Cocking, E.C. (1981). Improvement of legumes by exploiting extra specific genetic variations. Euphytica **30**:819-833.

Srivatanakul. M., Sanders. S. H. J. R, Salas, M. G. and Smith, R. H. (2000). Multiple shoot regeneration of kenaf from the shoot apex culture system. Plant Cell Rep. **19:** 1165-1170.

Skirvin, R.M. (1978). Natural and induced variation in tissue culture.

Euphytica. **27:** 24 1-266.

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

Zapata, C., Srivatanakul, M., Park, S. H., Lee, B. M., Salas, M. G. and Smith, R. H. (1999). Improvements in shoot regeneration of two fibre crops: cotton and kenaf. Plant Cell & Organ Cult. **56**: 185-191.

APPENDICES

APPENDICES

Appendix I. The composition of MS medium (Murashige and Skoog, 1962)

Macro nutrients	**Amount per liter (g)**
KNO_3	1.90
NH_4NO_3	1.650
$MgSO_4.7H_2O$	0.370
$CaCl_2.2H_2O$	0.440
KH_2PO_4	0.170
Micro nutrients	**Amount per liter (mg)**
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
Iron Source	**Amount per liter (mg)**
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Vitamins/Organics	**Amount per liter (mg)**
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Myo inositol	100 mg
Sucrose	30 g
Commercial Sugar	15 g
Agar	7 g

Appendix II. Analysis of variance of the data of media on number of seeds germination and percent seed germination

Source of variation	Degree of freedom	Number of seeds germinated	Percent seed germination
Treatment	1	28.167*	112.667*
Error	4	2.167	8.667

*= 5% significant level of probability

Appendix III : Analysis of variance of the data of effect of different days required for plant regeneration on percent shoot regeneration and number of shoot cotyledon

Source of variation	Degree of freedom	Number of explants showing shoot	Percent shoot regeneration	Number of shoot per cotydedon
Treatment	3	1.896*	1184.896*	0.437*
Error	9	0.785	490.451	0.139

*= 5% significant level of probability

Appendix IV: Analysis of variance of the data of effect of different concentration of vitamins on percent shoot regeneration, number of explants showing shoot and number of shoot per cotyledon.

Source of variation	Degree of freedom	Number of explants showing shoot	Percent shoot regeneration	Number of shoot per cotydedon
Treatment	4	0.875*	171.875*	0.392*
Error	12	0.292	442.708	0.122

*= 5% significant level of probability

Appendix V: Analysis of variance of the data of effect of different FeSO4 concentration on number of explants showing shoot , number of shoot per cotyledon and percent shoot regeneration

Source of variation	Degree of freedom	Number of explants showing shoot	Number of shoot per cotydedon	
Treatment	4	1520.833*	2.433*	0.203*
Error	8	333.333	0.533	0.040

*= 5% significant level of probability

Appendix VI: Analysis of variance of the data of effect of different concentration of surfactants (F-68) on percent shoot regeneration, number of explants showing shoot and number of shoot per cotyledon.

Source of variation	Degree of freedom	Percent of cotyledon producing shoot	Number of cotyledon producing shoots
Treatment	4	208.112**	31.049**
Error	8	1.065	0.062

**= 1% significant level of probability