DEVELOPMENT OF PLANT REGENERATION PROTOCOL FROM THE EXPLANTS OF MESTA *(Hibiscus sabdariffa* L.)

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CERTIFICATE

This is to certify that thesis entitled, "**Development of plant regeneration protocol from the explants of mesta (*Hibiscus sabdariffa* 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 **TAPAN KUMAR ROY**, Registration No.00343 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, 2006
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Dedicated to my beloved uncle

LIST OF ABBREVIATIONS

Abbreviation		Full word
%	:	Percentage
0.1 N	:	0.1 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
HCl	:	Hydrocloric acid
$HgCl_2$	:	Mercuric cloride
h	:	Hour (s)
IAA	:	Indol-3-acetic acid
IARI	:	Indian Agricultural Research Institue
IBA	:	Indol-3-butaric acid
ICRISAT	:	International Crop Research Institute for the Semi-arid Tropics
IRRI	:	International Rice Research Institure
j.	:	Journal
l	:	Litre
Lux	:	Unit of illumination
M	:	Molar or Manitol
min	:	Minute (s)
mg	:	Milligram
mg/l	:	Milligram per litre
ml		Millititre
ml/l	:	Millilitre per litre
MS	:	Murashige and Skoog (1962)
mW	:	Milliwatt (s)
N	:	Normal
Na_2-EDTA	:	Sodium salt of ferric ethyleve diamine tetraacetate
NAA	:	α-Napthelene acetic acid
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
No.	:	Number
NS	:	Non-significant
pH	:	Negative logarithm of hydrogen ion concentration (-log $[H^-]$)

Abbreviation		Full word
UK		United Kingdom
USDA	:	United States Department of Agriculture
UV	:	Ultra violet
var.	:	Variety
µg	:	Microgram

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The Author

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DEVELOPMENT OF PLANT REGENERATION PROTOCOL FROM THE EXPLANTS OF *MESTA (Hibiscus sabdariffa* L.*)*

By

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ABSTRACT

An experiment was conducted in the Genetic Engineering Laboratory of the Cytogenetics Department, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from January to July 2006. Cotyledons (with attached petioles) of mesta (*Hibiscus sabdariffa* L.*)* were used as explant to investigate their *in vitro* regeneration potentiality. Seed germination percentage was found highest on clinical cotton supported MS liquid medium (90%) than agar-based MS solidified medium (81.33%). Among the combination, MS medium + BAP (2.0 mg/l) + IAA (0.5 mg/l) showed the highest shoot regeneration (58.33%). No shoot regeneration was observed in MS media without hormone (MSO). Maximum shoot multiplication (46.67%) was found from shoot apex on MS medium + BAP (2.0 mg/l) + IAA (0.5 mg/l). MS medium with hormone and MS medium without hormone (MSO) were used for root formation from shoots. The highest number of initial roots per shoot (6.67) was found in the presence of IAA (2.0 mg/l). The maximum weight of roots per shoot (0.212g) was obtained in IAA (1.0 mg/l). The highest number of days to root initiation (9.00) was required in. IAA (0.5 mg/l) and the lowest (5.00) in IAA (0.0 mg/l) containing MS medium. The regenerated plantlets of mesta were successfully in soil.

CHAPTER I
INTRODUCTION

INTRODUCTION

Mesta (*Hibiscus sabdariffa* L.) is the best fibre crop next to jute and kenaf in importance. Mesta is called "roselle" in India, and in Thailand as "saimi jute". However it is called mesta in Bangladesh. It is a world wide adapted potential fibre and biomass producing crop. Mesta is a crop of tropical African origin and was domesticated in ancient times for its edible seeds subsequently for its leaves, young shoots and flower part as well (Maiti, 1997). As a vegetable, it reached America and Asia in the 17th Century. Nowadays mesta is widely distributed in the tropics and subtropics, usually in cultivation as a fibre plant or vegetables.

Hibiscus sabdriffa 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 other. Within *H. sabdariffa* two main cultivar groups are distinguished: Group Sabdariffa (*H. sabdariffa* var. *sabdariffa*): plants branch profusely, 1.5-2 m tall; epicalyx and calyx fleshy and glabrous, enlarging considerably after anthesis, edible; cultivated mainly as a vegetable. Group Altisima (*H. sabdariffa* var. *altissima* wester): plants with single stem, usually branching only at the top, 3.5-5 m tall; epicalyx and calyx leathery strongly hispid or bristled, enlarging little after anthesis, usually inedible; cultivated for its fibre.

Mesta is cultivated between 7°S of Java, Indonesia and 23°N of Bangladesh. Climatic requirements during the growing period are mean monthly temperatures of 25-30°C, a rainfall of 140-270 mm per month and high air humidity (> 70%).

The forgotten crops are *Hibiscus cannabinus* and *Hibiscus sabdariffa*, which in 1960 was selected by the United States Department of Agriculture from 500 crop species (which included hemp) 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 US into the field production and the paper making characteristics of mesta. All these information confirmed that mesta was a promising alternative feed stock to wood for pulp and paper production.

In the recent years, there has been an increasing trend in both area and production of mesta in Bangladesh. But the yield per unit area remains unsatisfactory. The average yield of mesta in Bangladesh is 1.9 t/ha which is much lower than that of many other mesta growing countries of the world such as India, Indonesia, China, Australia and USA (Ahmed and Vossen, 2003).

Mesta is usually propagated by seed, but can also be grown from stem cutting. Cultivar improvement in mesta follows breeding methods commonly applied to self-pollinating crops, such as line and pedigree selection after intervarietal crossing and backcrossing. In South-East Asia, the existing cultivars of mesta have a narrow genetic base and are constrained by low adaptability to agro-ecological conditions and susceptibility to several diseases and insects.

One of the major constrains to increase mesta 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. To improve the important agronomic characters of mesta, 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 developing crop cultivars, the difficulty in breaking gene linkages between useful and useless traits etc. In order to produce desirable lines of mesta

with high fibre yield, and higher biomass production an alternate technique is necessary.

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. Remarkable success have been demonstrated for the 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.

Plant tissue culture, a branch of biotechnology offers an efficient method for rapid propagation, production of pathogen free material and plant 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 mutagen induced genetic variation. These somaclonal variants in some cases can be useful for the selection of desirable genetic variants. Regenerants often display altered phenotypes, termed somaclonal variation (Larkin and Scowcroft, 1981). As part of the *Hibiscus cannabinus* improvement program at Mississippi State University, tissue culture is being employed as a means for introduction of new or altered traits into *Hibiscus cannabinus*. The exploitation of heritable somaclonal variants has been used in various plant improvement strategies (Larkin and Scowcroft, 1981; Evans, 1989; Larkin *et al.* 1989 and Phillips *et al.* 1994). Recently, plant regeneration has already been reported from the explants of mesta, which assures the exploitation of the species through tissue culture.

Improvement of a species through genetic engineering includes appropriate plant regeneration. 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 combinations and concentrations of hormone in the culture media (Ehsanpour and Jones, 2000; Fiegert *et al*. 2000 and Ahn *et al*. 2001).

The regeneration ability depends on optimum growth conditions, suitable explants and varieties. In the present investigation attempts were made to establish a suitable regeneration protocol for mesta.

The specific objective of this research programme was:

To develop a suitable and reproducible protocol for *in vitro* regeneration of mesta variety.

CHAPTER II
REVIEW OF LITERATURE

REVIEW OF LITERATURE

Mesta (*Hibiscus sabdariffa* L.) is one of the most important for fibre, biomass production and paper pulp yielding crop in the world, but the constraints of cultivation of this crop are insect infestation, disease infection and low yield. Pest hampers the production of *H. sabdariffa* cultivars in Bangladesh. To overcome this problem traditional breeding is employing in different countries including Bangladesh. But traditional breeding is time consuming and has a risk of assimilation of undesired genes, where plant tissue culture 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 genome.

2.1 *In vitro* regeneration of genus *Hibiscus*

2.1.1 Concept of tissue culture

The techniques of plant tissue culture have been developed as a new and powerful tools for crop improvement (Carlson, 1975 and Razdan and Cocking, 1981) and received wide attention of modern scientists (D'Aamato, 1978; Skirvin, 1978; Larkin and Scowcroft, 1982).

Regeneration from different explants (leaf, stem, cotyledon, hypocotyls root etc.) on defined nutrient media under sterile condition 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 regeneration.

Nowadays, plant tissue culture techniques have been emerged as a world wide accepted concept 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* culture 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 genus *Hibiscus*

In vitro regeneration has been quite difficult among the species of mesta, kenaf and jute through tissue culture technique. It appears that mesta and kenaf is a 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 observed from cotyledons with attached petioles and hypocotyls where meristematic tissue present.

As part of the *Hibiscus cannabinus* improvement program at Mississippi State University, Larkin and Scowcroft (1981) employed tissue culture as a means for introduction of new or altered traits into *H. cannabinus*. Regenerations 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) and Phillips *et al.* (1994). But success has been sporadic. Reichert and Liu (1994) has optimized adventitious shoot regeneration protocols for starting with internodal stem and leaf sections. They obtained plant regenerated three new varieties: Everglades 41 (E41), Guatemala 45 (G45), and G48.

2.1.2.1Callus induction

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 hormone have an influence on callus induction from various *Hibiscus cannabinus* varieties. The most relevant literatures related to callus induction have been reviewed here. Various explants like cotyledons with attached petioles, hypocotyles containing meristemetic zones and shoot apices were used for regeneration from *Hibiscus cannabinus*.

McLean *et al.* (1992) first reported regeneration from kenaf plants *in vitro* at Mississippi State University. They cultured Internodal stem explants on media containing different combinations and concentrations of auxins and cytokinins (PGR's). Within 5 days, callus formed around the periphery of the explants.

Adventitious shoots developed from the callus within 30 days. The shoots were excised and placed on a different medium for root formation.

2.1.2.2 Shoot regeneration

A direct and simple regeneration procedure using shoot apex is reported by Zapata *et al.* (1999). Regeneration from internodal section was reported by Reichert and Liu (1994). Internodal stem sections were taken from field-grown plants of 'Everglades 41' ('E41'), were cultured on Murashige and Skoog (1962) salts supplemented by 1-Naphthalene acetic acid (0.1 mg/l) (NAA) and Thidiazuron (TDZ) (3.0 mg/l) for shoot initiation.

Purwati and Sudarmadji (1999) studied the response of five *Hibiscus cannabinus* accessions for shoot regeneration and established regeneration protocol for *Hibiscus cannabinus*. From cotyledons with attached plumules Purwati *et al.*

(1999) used MS based medium containing BAP (2 mg/l) and GA_3 (0.5 mg/) for callus induction. Calli produced in this culture were than transferred into MS-based medium containing BAP (2 mg/l) and GA_3 (5 mg/l) for shoot initiation.

Khatun (2001) observed multiple shoots from cotyledons with attached petioles explants of *C. capsularis* on MS medium supplemented with IAA (0.5 mg /l) and BAP (2.0 mg/l).

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* 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 *Hibiscus cannabinus* cotyledons with attached petioles *in vitro*. The effect was the 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.

In vitro regeneration method is also reported in *Hibiscus cannabinus* by Herath *et al.* (2004). Multiple shoots induced from shoot tips and cotyledonary nodes of *Hibiscus cannabinus* cultured on MS medium treated with Benzyl Adenine (BA). Herath *et al.* (2004) reported that the number of shoots regenerated varied with the cultivars, explants, and the BAP concentration. Highest number of shoots (11/explant) was developed in cultivar Tainung 2 (T 2) shoot tips cultured in MS medium supplemented with BA (2.0 mg/l).

Srivatanakul *el al.* (2000) developed a medium that stimulate multiple shoot initiation from explants of kenaf (*Hibiscus cannabinus*). Adventitious shoot

formation on a shoot induction medium supplemented with combinations of 2,4-D (0, 0.5 and 2.3 μ mol/l) and Thidiazuron (0, 1, 5 and 20 μ mol/l) was evaluated. Multiple shoot induction medium with 1 μ mol/l TDZ/l resulted the highest number of regenerated shoot per explant.

2.1.2.3 Root induction

Jute (*Corcorus capsularis)* and mseta (*Hibiscus sabdariffa*) both are fiber plants. *In vitro* root induction of jute was reported by several researchers.

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.

Saha *et al.* (1999) reported that the best root formation induced in the MS medium with 2.5 μ MIBA and 1.5 % sucrose.

Ahmed *et al.* (1989) reported that the *in vitro* regenerated shoots of *C. olitorius* (var. 0-4) produced roots most successfully on MS medium with 3 mg nirdihydrogniaretic acid + IBA (0.3 mg/l).

CHAPTER III
MATERIALS AND METHODS

MATERIALS AND METHODS

The present investigation was carried out in the Genetic Engineering Laboratory of the Department of Genetic Resource and Seed Division, Bangladesh Jute Research Institute from January to June 2006. These experiments were conducted to fulfill the objective of the present study.

Experiment 1: *In vitro* seed germination potentiality of mesta (*Hibiscus sabdariffa)*

Experiment 2: *In vitro* shoot regeneration from cotyledon with attached petioles of *Hibiscus sabdariffa*

Experiment 3: *In vitro* shoot multiplication from shoot apex of *Hibiscus sabdariffa*

Experiment 4: *In vitro* Root induction from the regenerated plantlets and shoots of mesta.

Experiment 5: Survivability of plantlets into soil

3.1 Experimental Materials

3.1.1 Sources of explants for tissue culture

Mesta (Var. HS-24) seeds used for seedling production in this experiment were collected from Bangladesh Jute Research Institute (BJRI), Dhaka.

3.1.2 Plant materials

Cotyledons with attached petioles of mesta (var. HS-24) were used for regeneration.

3.1.3 Media Used

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

3.1.3.1 Medium used for seed germination

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

3.1.3.2 Medium used for shoot regeneration

Agar based MS (Murasighe and Skoog, 1962) solidified medium supplemented with IAA (0.5 mg/l) and BAP (0.25, 0.5, 2, 3 and 4 mg/l) was used for plant regeneration. MS medium without hormone was used as control.

3.1.3.3 Medium used for Root initiation

MS medium with hormone (IAA) and MS medium without hormone (MSO) were used for root production.

3.2 Methods

3.2.1 Media preparation

3.2.1.1 Preparation of stock solutions

For the preparation of different stock solutions for macronutrients, micronutrients, iron, vitamins, hormones were prepared and stored appropriately for use. 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 solutions stored in a refrigerator at 4^0C 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 later use.

iii) Iron source C

The required amount of $FeSO_4$ was weighted and added directly 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 and stored at 20^0C. Myo-inositol 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 powder 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.

3.2.1.2 Steps followed for the preparation of culture media

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

3.2.1.2.1 Preparation 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 2litre Erlenmeyer flask on a magnetic stirrer.

ii) Four hundred and fifty ml distilled water was added in the flask to dissolve all the ingredients.

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

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

v) 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.

vi) P^H of the medium was adjusted to 5.8 with a digital p^H meter by adding NaOH or HCl (1% solution) whichever was necessary.

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

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

ix) 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.

3.3 Sterilization techniques

3.3.1 Sterilization of culture media

All prepared media were autoclaved at 15psi 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 glasswares and instruments

Beakers, test tubes, conical flasks, pipettes, small instrument like forceps, scalpels, micropipette, were prepared for autoclaving 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 121^0C 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. Generally, 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 tube inside the cabinet. Both of the hands were rinsed with 95% alcohol. All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants.

3.4 Culture techniques

The following culture techniques were employed in the present investigation:

i) *In vitro* seedling raising

ii) Cotyledon culture

iv) Multiplication of shoot from shoot apex

iii) Shoot culture for root induction

v) Transfer to soil

3.4.1 *In vitro* seedling raising

Healthy seedling production was found to be one of the major criteria for plant regeneration from *Hibiscus sabdariffa* explants and thereby to be successful in genetic transformation. Seeds of *H. sabdariffa (var. HS-24)* 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 sterilized 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°C temperature under 1500 lux fluorescent illumination with 12 h photoperiod.

3.4.2 Cotyledon culture

Cotyledons with attached petioles were taken form *in vitro* raised *H. sabdariffa* (var. HS-24) seedlings. 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 cultured on MS medium in 250 ml 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/l) and BAP (0.0, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) were used. The cultures were maintained in a growth room with 27±2°C temperature under 1500 lux fluorescent illumination of 12 h photoperiod. 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 after 35 days of culture.

3.4.3 Multiplication of shoot from shoot apex

Shoot apices were taken from *in vitro* raised *H. sabdariffa* seedlings. Seedlings were used for shoot apices culture. 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/l) and BAP (1.0, 2.0 and 4.0 mg/l) were used. The cultures were maintained in a growth room with 27±2°C temperature under 1500 lux fluorescent illumination of 12 h photoperiod. The conical flasks were checked daily to note the response and the development of contamination. Number of shoots multiplied from shoot apices of *H. sabdariffa* in the culture medium was recorded after 45 days of culture.

3.4.4 Shoot culture for root induction

When the regenerated shoots were 2-3 cm in length, they were rescued aseptically from the cultured flasks and were separated from each other and again cultured individually on 250 ml conical flask with freshly prepared MS medium without hormone (MSO) for root initiation.

In the second set of experiments the shoots (2-3 cm) of mesta seedlings were cultured on MS medium supplemented by different IAA (0.0,0.5,1.0 and 2.0 mg l). The shoots were vertically placed and gently pressed on the surface of the sterilized culture medium so that the cut ends of the shoots were inserted into the medium to a depth of 8-10 mm for root production.

The conical flasks containing plantlets and shoot were cultured at 28^0 C under a 1.0 Wm^{-2} of daylight fluorescent tubes with a 12 h photoperiod. Day to day observations was carried out to note the responses.

3.4.5 Transfer of plantlets into soil

Mesta plantlets were transferred into pots containing mixed soil. Saver Dairy soil (70%) was mixed with 30% commercial sand. The mixture was sterilized before use. The idea of using mixture was to make the soil porous and aerated. The earthen pots (6 cm dia. and 7 cm height) with a small hole at the bottom were used for transfer of plantlets. Pots were placed on a 9 cm petri dishes each containing 20 ml of water. The root of plants were washed with sterilized tap water to remove agar and then transferred into the pots. The plantlets were then covered with a cellophane paper bag and placed in a well ventilated room. After one week, two holes were made in the bags. During the third week the bags were removed. Survival rate of the plants was recorded.

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 from the different parameters as given below:

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.

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

3.5.2 Percent Shoots regeneration

The number of cotyledons producing shoot and the number of shoots per cotyledon were recorded. The percentage of shoot regeneration was calculated on the basis of the number of cotyledons cultured and the total number of cotyledons producing shoot.

$$\text{Percent shoot regeneration} = \frac{\text{No. of cotyledons producing shoot}}{\text{No. of cotyledons cultured}} \times 100$$

3.5.3 Multiplication of shoots

The number of shoot apices producing multiplied shoots and the number of multiplied shoots per shoot apex were recorded. The percentage of shoot multiplication were calculated on the basis of the number of shoots apices cultured and the total number of shoot apices producing multiplied shoot.

$$\text{Percent shoot multiplication} = \frac{\text{No. of shoot apices producing multiplied shoot}}{\text{No. of total shoots apices cultured}} \times 100$$

3.5.4 Root induction

Generally, root initiation started at 6-9 days of cultured shoots. The number of days was recorded for root initiation in different IAA concentrations. The number of initial roots and the weight of roots per shoot were recorded.

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

Healthy seedling production was one of the major criteria for plant regeneration from mesta explants. Seeds of mesta (var. HS-24) germinated on both clinical cotton-supported MS liquid medium and agar-based MS solidified medium.

4.1 Experiment 1: *In vitro* seed germination potentiality of mesta (*Hibiscus sabdariffa)*

4.1.1 Percent seed germination

Percent seed germination from mesta variety was found to be higher on cotton-supported MS liquid medium (90.00%) compared to agar-based MS solidified medium (81.33%) (Table1). Growth of the seedlings in cotton-supported MS liquid medium was found to be comparatively better than agar-based MS solidified medium. This finding is similar to the finding of Khatun (2001) who reported that germination percentage of jute was found higher in cotton-supported MS liquid medium than the agar-based MS solidified medium.

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

Cotton vs. agar based medium	Number of germinated seeds	Percent seed germination
Cotton supported MS liquid medium	45.00a	90.00a
Agar based MS solidified medium	40.67b	81.33b
Level of significance	*	*
LSD 0.05	3.33	6.67

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

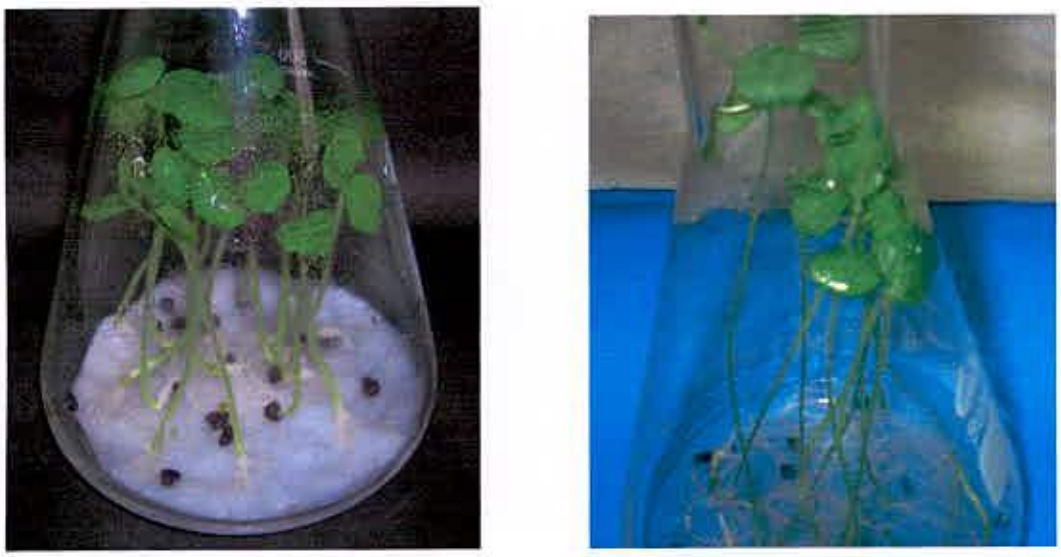

Plate 2. Seed germination on clinical cotton-supported MS liquid (left) and agar-based MS solidified medium (right)

4.2 Experiment 2: *In vitro* shoot regeneration from cotyledon with attached petioles of *Hibiscus sabdariffa*

4.2.1 Percent shoot regeneration

Percent shoot regeneration of mesta in IAA (0.5 mg/l) with different concentrations of BAP (0.25, 0.50, 1.0, 2.0 and 3.0 mg/l) was presented in Table 2. The highest regeneration potentiality (58.33%) was observed in IAA (0.5 mg/l) with BAP (2.0 mg/l) concentration and the lowest shoot regeneration percent (44.44%) was observed in IAA (0.5 mg/l) with BAP (0.5 mg/l) (Table 2). Regarding shoot regeneration, all the treatments statistically similar except BAP (4.0 mg/l) + control.

Shoot regeneration was restricted to the proximal cut end of petioles. This finding was consistence with the results for *Brassica juncea* (Sharma *et al.* 1991) jute (Khatun *et al.* 1993) and kenaf (Khatun *et al.* 2003)

This observation is similar to the finding of Khatun (2001) who reported that the highest number of regenerated shoots were obtained from cotyledons with attached petioles explants of *C. capsularis* on MS medium supplemented by IAA (0.5 mg /l) and BAP (2.0 mg/l). In both the experiments explants and hormone combination and concentrations are similar. However, no shoot regeneration was observed without hormone (Table 2)

4.2.2 Shoots per cotyledon

The highest number (4) of shoots per cotyledon was obtained at BAP 2 mg/l with IAA 0.5 mg/l (Table 2). The lowest number (1.67) of shoots per cotyledon obtained in MS medium supplemented with BAP 1.0 mg/l and IAA 0.5 mg/l (Table 2).

Table 2. Effect of different hormone concentrations on average number of cotyledons producing shoot, percent shoot regeneration and number of shoots per cotyledon

IAA (mg/l)	BAP (mg/l)	Average number of cotyledons producing shoos	Percent shoot regeneration	Number of shoots per cotyledon
	0.00	0.00b	0.00b	0.00c
	0.25	5.67a	47.22a	2.17b
	0.50	5.33a	44.44a	2.33b
0.50	1.00	6.00a	50.00a	1.67b
	2.00	7.00a	58.33a	4.00a
	3.00	6.00a	50.00a	3.67a
	4.00	0.00b	0.00b	0.00c
Level of significance		**	**	**
LSD 0.01		1.576	13.13	1.064

Plate 3. Cultured cotyledons on MS medium supplemented by IAA (0.5 mg/l) and BAP (2.0 mg/l)

Plate 4. Shoot regeneration from cotyledons with attached petioles on MS medium supplemented by IAA (0.5 mg/l) and BAP (2.0 mg/l)

Plate 5. Shoot regeneration from cotyledons with attached petioles in MS medium supplemented by IAA (0.5mg/l) and BAP (3.0mg/l)

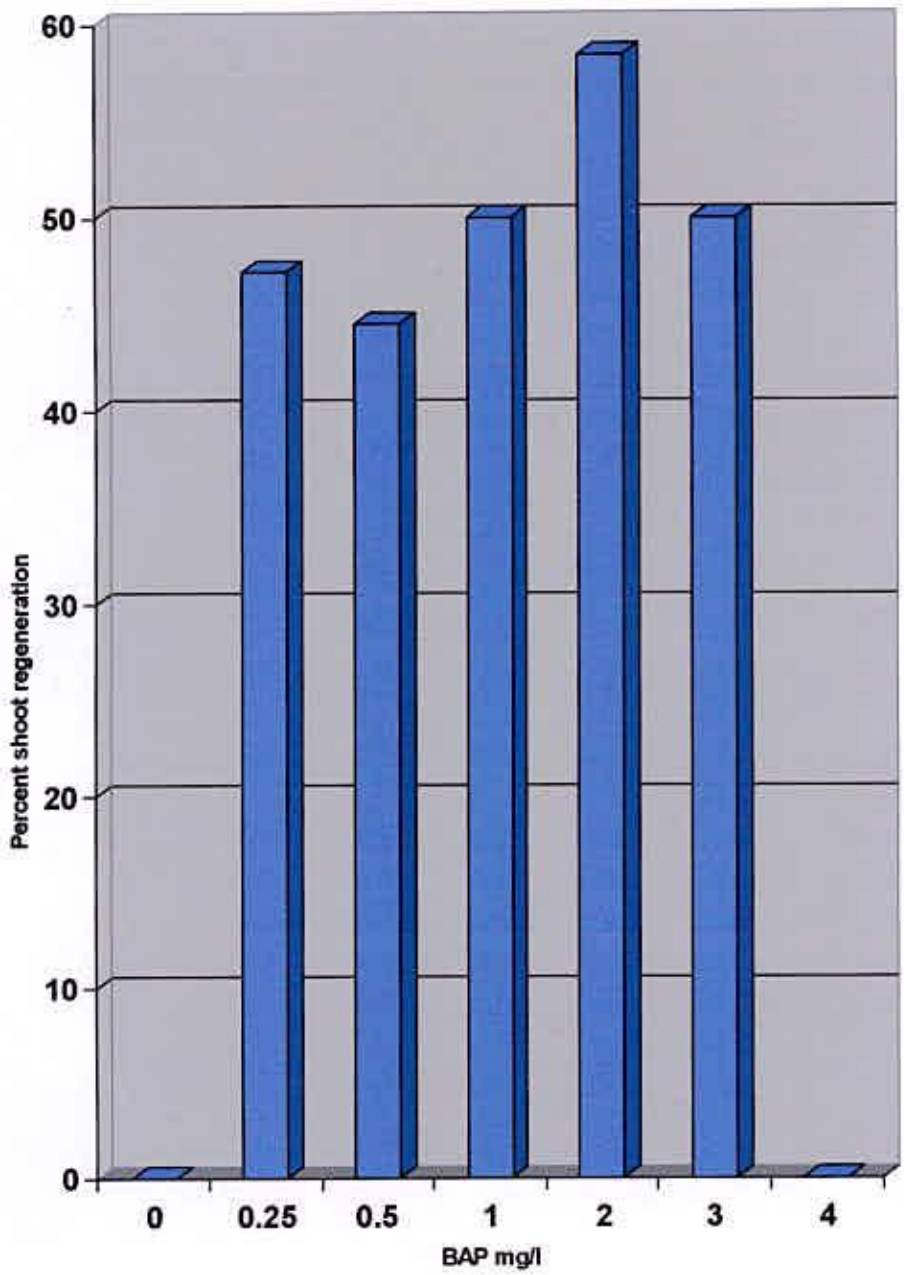

Fig. 1. Effect of different BAP concentrations on percent shoot regeneration from mesta cotyledons attached with petioles, IAA (0.50mg/l) was used in all cases

4.3 Experiment 3: *In vitro* shoot multiplication from shoot apex of *Hibiscus sabdariffa*

4.3.1 Effect of hormone (IAA)

Two IAA concentrations were used in this experiment. IAA at 0.50 mg/l concentration showed better performance for shoot multiplication. Maximum number of shoot apices produced multiplied shoots (3.56) with the highest percentage of shoot multiplication (29.53%) at IAA (0.5 mg/l) (Table 3).

4.3.2 Effect of hormone (BAP)

Different concentrations of BAP showed significant variation for number of shoot apex producing multiplied shoots and percent shoot multiplication. BAP at 2.0 mg/l was found to be the best for multiplication (Table 4).

4.3.3 Combined effect of BAP and IAA

Combined effect of BAP and IAA on number of shoot apices producing multiplied shoots, number of shoots per shoot apex and percent shoot multiplication are presented in the Table 5.

The highest percentage of shoot multiplication (46.67%) was found in the combination of MS+ IAA (0.5 mg/l) + BAP (2 mg/l). Maximum number of shoot apices producing multiplied shoots (5.67) was also observed in the same combination. (Table 5).

Shoot multiplication from shoot apices of *C. capsularis* and *C. olitorius* was observed by Khatun (1993). She reported that BAP (1.0 mg/l) with IAA (0.5 mg/l) was required for shoot multiplication. This hormone combination (IAA and BAP) which was found similar to mesta. However, unlike jute, BAP requirement was higher (3.0 mg/l) for mesta.

Table 3. Effect of different concentrations of IAA on number of shoot apices producing shoots, percent shoot multiplication and number of multiplied shoots per shoot apex

Treatment (IAA/l)	Number of shoot apices producing shoots	Percent shoot multiplication	Number of multiplied shoots per shoot apex
0.50	3.56	29.53	1.89
1.00	3.33	27.78	1.56

Table 4. Effect of different concentrations of BAP on number of shoot apices producing shoots, percent shoot multiplication and number of multiplied shoots per shoot apex

Treatment (BAP/l)	Number of shoot apices producing shoots	Percent shoot multiplication	Number of multiplied shoots shoot per apex
1	3.17	1.50	26.39
2	4.50	2.17	37.50
3	1.15	1.50	22.22

Table 5. Combined effect of different concentrations of IAA and BAP of number of shoot apices producing shoots, Percent shoot multiplication and number of multiplied shoots per shoot apex

IAA (mg/l)	BAP (mg/l)	Number of shoot apices producing shoots	Percent shoot multiplication	Number of multiplied shoots shoot per apex
0.5	1	1.00b	8.33b	0.67b
0.5	2	5.60a	46.67 a	2.67a
0.5	4	4.33a	35.11a	2.33a
1.0	1	5.33a	44.44a	2.33a
1.0	2	3.67a	30.55a	1.67ab
1.0	4	1.00b	8.33b	0.67b
Level of significance		**	**	**
LSD 0.01		1.625	13.53	1.027

Plate 6. Multiplied shoots obtained from mesta shoot apices in MS medium supplemented by IAA (1.0 mg/l) and BAP (2.0 mg/l) at 45 DAC (days after culture)

Plate 7. Multiplied shoots obtained from mesta shoot apices in MS medium supplemented by IAA (1.0 mg/l) and BAP (1.0mg/l) at 45 DAC (days after culture)

4.4 Experiment 4: *In vitro* Root induction from shoots and the regenerated plantlets of mesta.

Root initiation was observed from cultured shoots of mesta on different IAA concentrations (0.0, 0.5, 1.0 and 2.0 mg/l). Different IAA concentrations showed significant variation in root production. (Table 6). Regenerated shoots were transferred on MS medium without hormone (MSO) for root production. Root induction was observed from the transferred shoots.

4.4.1 Root production per shoot

Number of initial roots mainly depends on different IAA concentrations. The highest number of initial roots per shoot (6.67) was obtained in (IAA 2.0 mg/l) and the lowest number (2.0) in MS medium without hormone (MSO) (Table 6).

4.4.2 Weight of root per shoot

Different concentrations of IAA showed significant variation for root weight (g) per shoot. The highest root weight per shoot (0.212 g) was obtained in IAA 1.00 mg/l and the lowest weight (0.051g) in MS medium without hormone (MSO) (Table 6).

4.4.3 Days to root initiation

Days required for root induction was found to be dependent on hormone concentration used. It was observed that minimum days required for root initiation was 5 on MS medium without hormone (MSO). This finding is similar to the finding of Khatun (2001) who reported that the shoot of *C. olitorius* and *C. capsularis* were rooted on MS medium without hormone within 7 days.

The maximum days required for root initiation was 9 on MS medium supplemented by IAA (0.5 mg /l) (Table 6). Hormone was also need for root induction from the shoots of jute (Saha *et al.* 1999), *C. olitorius* (var. 0-4) (Ahmed *et al.* 1989), okra (Roy and Mandat 1988) and carambola (Khalekuzzaman *et al.*, 1995) which is in agreement with the present study. However, unlike mesta, MIBA was used for jute, nirdihydrogniaretic acid + IBA for *C. olitorius* (var. 0-4), NAA for okra and IBA for carambola.

Table 6. Effect of different concentrations of IAA on initial roots per shoot, weight of roots per shoot and days to root initiation

Treatment (IAA mg/l)	Number of initial roots per shoot	Weight of roots per shoot	Days to root initiation
0.00	2.00c	0.051c	5.00c
0.50	3.67b	0.161b	9.00a
1.00	4.33b	0.212a	7.33b
2.00	6.67a	0.173b	5.33c
Level of significance	**	**	**
LSD 0.01	1.331	0.019	1.538

Plate 8. Root production from shoots of mesta on MS medium without hormone (MSO) at 35 DAC (days after culture)

Plate 9. Root production from shoots of mesta on MS medium supplemented by (IAA 0.5 mg/l) at 35 DAC (days after culture)

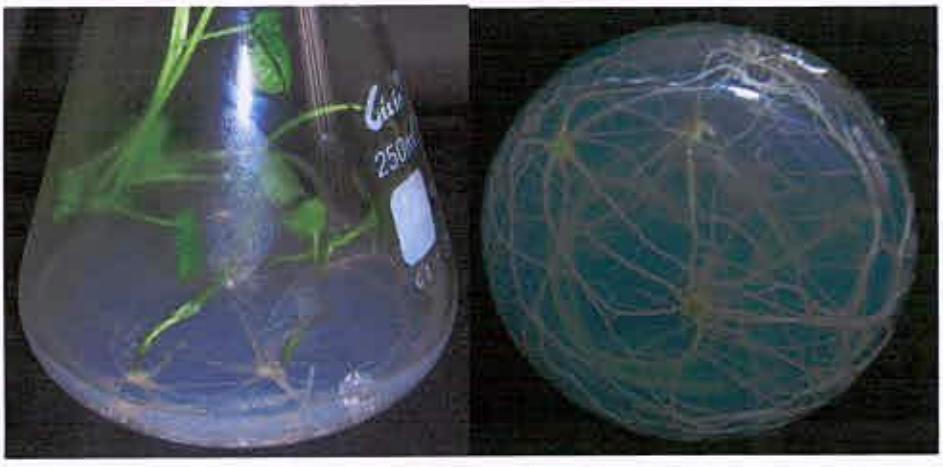

Plate 10. Root production from shoots of mesta on MS medium supplemented by IAA (1.0 mg/l) at 35 DAC (days after culture)

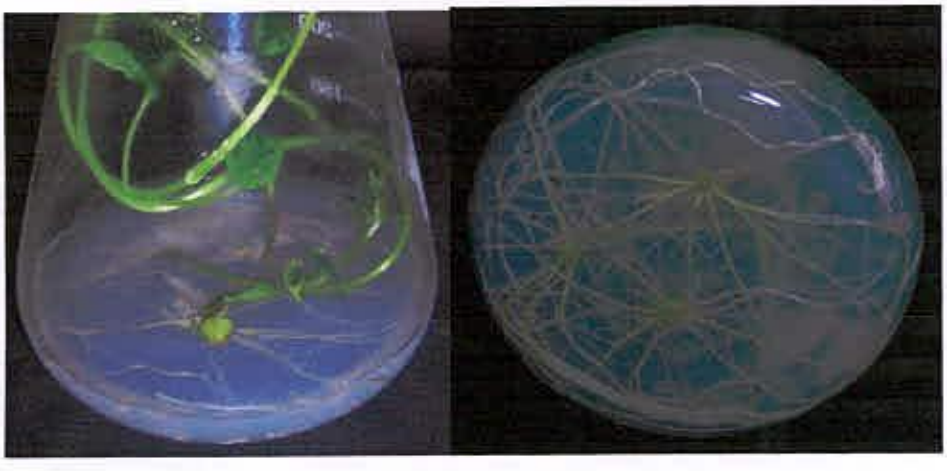

Plate 11. Root production from shoots of mesta on MS medium supplemented by IAA (2.0 mg/l) at 35 DAC (days after culture)

Plate 12. Initiation of roots from regenerated shoot of mesta on MSO medium

4.5 Experiment 5: Survivability of plantlets into soil

Root production was observed on hormone free MS medium. Plantlets were successfully transferred to mixed soil (dairy soil and sand). Ninety five percent mesta plantlets survived after transfer to soil and grew up to maturity.

Plate 13. Regenerated plantlets of mesta were covered with cellophane bag after transferred into soil

Plate 14. Successfully transferred plantlets at 15days after transferred into soil

CHAPTER V
SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Experiments were carried out in the Genetic Engineering Laboratory, Cytogenetics Department, Bangladesh Jute Research Institute (BJRI), Dhaka, during the period of January to June 2006.

In the experiments, detailed investigation was carried out to study the seed germination, shoot multiplication, plant regeneration and root initiation of mesta (HS-24) genotype using cotyledons (with attach petioles), shoot apices and shoots as explants.

Significant effect was found in percent seed germination. In cotton-supported MS liquid medium, the germination percentage was found to be higher (90.00%) compared to agar-based MS solidified medium (81.33%). From the results of the present study, it was observed that cotton supported MS liquid medium was better than agar-based MS solidified medium for seed germination.

Cotyledons (with attached petioles) of mesta were cultured on MS medium supplemented by various BAP concentrations and without hormone to observe the shoot regeneration performance of cotyledons. From the study, different hormone concentrations showed different regeneration performance. The highest percentage of shoot regeneration (58.33%) was found on MS medium + IAA (0.5 mg/l) with BAP (2.0 mg/l) and the lowest (44.44%) on MS medium + IAA (0.5 mg/l) with BAP (0.5 mg/l). No regeneration was obtained on MS medium without hormone (MSO).

Different concentrations of BAP and IAA on MS media were used to observe shoot multiplication capacity from shoot apex. It appeared from the study that the responses of different levels of hormone concentrations were different. The highest percentage of shoot multiplication (46.67%) was found in the combination of MS medium + IAA (0.5 mg/l) with BAP (2.0 mg/l) (46.67%) followed by MS medium + IAA (1.0 mg/l) with BAP (1.0 mg/l) (44.44%). Maximum number of shoot apices showing shoot (5.67) was observed in the combination of MS medium + IAA (0.5 mg/l) with BAP (2.0 mg/l).

Different concentrations of IAA on MS medium with or without hormone were used to observe the root initiation capacity of shoot. It appeared from the present study that the responses of different levels of IAA were different. The highest number of initial roots per shoot (6.67) was found on MS medium + IAA (2.0 mg/l) and the lowest (2.00) on MS medium without IAA. The maximum weight of roots per shoot (0.212g) was obtained on MS medium + IAA (1.0 mg/l) and minimum (0.051) on MS medium without IAA. The highest number of days to root initiation (9.00) was observed on MS medium + IAA (0.5 mg/l) and the lowest (5.00) on MS medium without IAA. Regenerated shoots were transferred to MS medium without hormone for root production, then the shoots were transferred to mixed soil. Maximum percentage of plantlets grew into maturity.

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

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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

Appendix III. Analysis of variance of the data of number of cotyledons producing shoots, number of shoots per cotyledon and percent shoot regeneration

Source of variation	Degree of freedom	Number of cotyledons producing shoot	Percent shoot regeneration	Number of shoots per cotyledon
Treatment	6	26.512**	7.512**	1839.727**
Error	14	0.810	0.369	56.217

Appendix IV. Analysis of variance of the data of different concentrations of IAA and BAP on number of shoot apices producing multiplied shoots, percent shoot multiplication and number of multiplied shoots per shoot apex

Source of variation	Degree of freedom	Number of shoot apices producing multiplied shoots	Percent shoot multiplication	Number of multiplied shoots per shoot apex
Concentration of IAA (A)	1	0.222	0.500	15.432
Concentration of BAP (B)	2	5.389**	0.889	374.228
Interaction (A×B)	2	24.389**	4.607**	1693.673**
Error	12	0.556	0.333	38.580**

Appendix V. Analysis of variance of the data of initial roots per shoot, weight of roots per shoot and days to root initiation

Source of variation	Degree of freedom	Initial roots per shoot	Weight of roots per shoot	Days to root initiation
Treatment	3	11.222**	0.014**	10.444**
Error	8	0.500	0.0001	0.666

** = 1% significant level of probability

* = 5% significant level of probability