OPTIMIZATION OF *IN VITRO* PLANT REGENERATION PROTOCOL FROM COTYLEDONS OF TOSSA JUTE (*Corchorus olitorious*)

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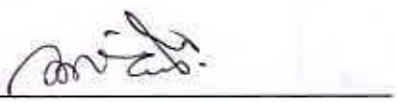

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CERTIFICATE

*This is to certify that the thesis entitled, "**Optimization of In vitro Plant Regeneration Protocol from Cotyledons of Tossa Jute (Corchorus olitorius)**" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfilment 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 **KAZI MD. KAMRUL HUDA**, Registration No. 25208/00334 under my supervision and my 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 been duly acknowledged by him.

Dated: June, 2006
Dhaka, Bangladesh

(Professor. Dr. Md. Shahidur Rashid Bhuiyan)
Supervisor

Dedicated To My Beloved Parents

LIST OF ABBREVIATIONS

Abbreviation		Full word
%	:	Percentage
0.1 N	:	0.1 Normal
BAP	:	6-benzyl amino purine
BBS	:	Bangladesh Bureau of Statistics
CaMV	:	Cauliflower Mosaic Virus
CIP	:	International Potato Centre
DMRT	:	Duncan's Multiple Range Test
dw	:	Distilled Water
e.g.	:	Exempli gratia (by way of example)
et al.	:	et alu=other people
etc.	:	et cetera (means and the rest)
g	:	Gram
gl^{-1}	:	Gram per litre
GUS	:	B-glucuronidase
HCl	:	Hydrocloric acid
$HgCl_2$	:	Mercuric Cloride
hrs.	:	Hours
i.e.	:	ed est (means That is)
IARI	:	Indian Agricultural Research Institute
ICRISAT	:	International Crop Research Institute for the Semi-arid Tropics
IRRI	:	International Rice Research Institute
j.	:	Journal
Kan	:	Kanamycin
LB	:	Luria Broth
mgl^{-1}	:	Milligram per litre
ml	:	Millititre
MS	:	Murashige and Skoog
Na_2-EDTA	:	Sodium salt of ferric ethylene diamine tetraacetate
NAA	:	α-napthelene acetic acid
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
No.	:	Number
nptII	:	Neomycin phosphotransferase II
NS	:	Non-significant
pH	:	Negative logarithm of hydrogen ion concentration (-log $[H^+]$)
req.	:	Required

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Dated:
Dhaka,Bangladesh.

The Author

OPTIMIZATION OF *IN VITRO* PLANT REGENERATION PROTOCOL FROM COTYLEDONS OF TOSSA JUTE (*Corchorus olitorious)*

By

KAZI MD. KAMRUL HUDA

ABSTRACT

Sets of experiments were conducted in the Genetic Engineering Laboratory, Cytogenetic Department, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from March 2005 to May 2006 to fulfill the objectives of the study. One of the major constraints of getting plant regeneration from the explants of *C. olitorious* was the production of healthy seedlings *in vitro*. Seeds of *C. olitorious* germinated on both agar supported hormone free MS medium and cotton supported hormone free liquid MS medium. The percentage of seeds germinated on cotton supported medium was found to be much higher than seeds germinated on agar supported medium. This system ensured maximum of an average of 97.55% seed germination. Whereas, in case of agar supported medium an average of 45.8% seeds could be germinated with very slow growth. The seedling grown on cotton supported medium were found to much more healthy than seedlings grown on agar supported medium. Plant regeneration obtained from the cotyledonary petioles of the varieties of *C. olitorious* on MS agar solidified medium supplemented by IAA 0.5 mg/l and BAP 3.0 mg/l. Plant regeneration was also observed in different level of P^{II} and different concentration of $FeSO_4$ and it was found that 5.5 and 56 mg/l gave highest result. The efficiency of plant regeneration from the cotton supported seedlings was found to be better as the agar supported seedlings. The plantlets produced roots on hormone free MS medium rapidly. After transfer to soil the plants grew into maturity and produced fruits. No morphological changes were noticed.

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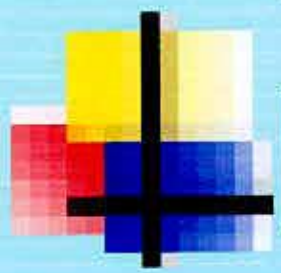

INTRODUCTION

Jute is one of the major world fiber crops, which is particularly important to the economy of Bangladesh, where the bulk of the world's supplies are grown. The two cultivated species *Corchorus capsularis* and *Corchorus olitorius* (2n=14), belonging to the family of Tiliaceae yield a bast (bark) fibre which is one of the most important vegetative fiber next to cotton (Singh, 1976). Commonly *C. olitorius* is known as tossa jute and *C. capsularis* as white jute. Jute is not only a major foreign currency earner, but also a major source of employment for the producer countries. It is of prime importance in the rural economy of the regions in which it is grown.

Jute is one of the most important cash crops of Bangladesh. It occupies 5th position after rice, pulses, oil seeds and wheat in respect of cultivated area (BBS, 2005). Bangladesh is not only the second largest producer of jute but she produces the best quality jute and leads the export market. In the year of 2003-2004, the acreage, production and yield of jute was 1128 thousand acre, 859 thousand bales and 762 kg/acre respectively (BBS, 2005). It is extensively used in the manufacture of different types of packing materials for various agricultural and industrial products.

Commercially jute is often referred to as the "golden fiber of Bangladesh", because of its immense contribution for the economy of this country. Considerable size of the total population of our country is engaged directly and indirectly in production and processing of jute. Jute exports constitute a major source of foreign exchange (12-13%) earning in Bangladesh. During the

year of 2004-2005 Bangladesh exported 619000 tons of raw jute and jute goods and earned about 16908 million Taka. (BBS, 2005).

Cultivation of jute in Bangladesh is increasingly shifting to less productive land with marginal care. Thus creating challenges in dealing with new emerging production constraints. In every year about 7 lakh 67 thousand bales of jute are damaged by insect-pest (Ahmed *et al.* 1993). Diseases have also an adverse effect on yield. A biotic stresses like drought, flood and low temperature etc are detrimental to this crop. With the launching of global campaign for environmental awareness international opinion is being created on jute for its expanded production and use, as it is biodegradable and friendly to the environment. Jute is a plant, all parts of which have extensive uses. Sustainable improvement in jute productivity under less favorable environment can only be achieved with a constant flow of new genetic materials. The existing variable for constraints, like insect-pest and diseases, poor soil fertility, water stress, fiber quality, photo-insensitive etc. is a serious issue that needs to be addressed (Agarwal, 2000). One of the major constraints to increase jute productivity is the non-availability of modern varieties with improved plant types.

Modern biotechnological techniques may also be utilized conveniently to overcome incompatibility barrier through fusion of protoplasts from vegetative cells of inter-specific, inter-generic and interfamilial group. However in several instances, callus derived from fused protoplasts could not be induced to regenerate plantlets (Rao and Chadha 1986, Rao 1985). Transformation of higher plants has been accomplished by different methods (Gardner 1993, Paszkowski *et al.*, 1989). The most common and efficient one utilizes non-oncogenic *Agrobacterium* strain as a gene vector. From this information, it is

evident that all the tissue culture techniques play a vital role in the enrichment of genetic variability. This particular technique contributed a little in the production of disease and pest resistant plants as well as plants of better agronomic characters in jute.

At present not much success can be achieved for jute production through the conventional breeding methods. New genotypes are, therefore, very much for improving yield potentiality. Although considerable number of high yielding varieties of jute has been released from the Bangladesh Jute Research Institute (BJRI) through conventional breeding techniques, these techniques still have some limitations. It is therefore, very important to explore other means of modern scientific techniques for example, tissue culture or genetic engineering to accelerate the pace of varietals improvement.

The chances for availability of new genotype of jute with disease resistance in nature are very remote unless new techniques are launched to create variability. Biotechnology is a recently developed novel approach and therefore it is very important to explore these techniques for varietal improvement of jute.

The pre-requisite for the genetic transformation in jute is to establish an efficient system of plant regeneration from explants to matured fertile plants. Plant regeneration from the cotyledon petioles reported earlier from *C. capsularis* (Khatun *et al.*, 1992) and from the shoot apices of *C. olitorius* (Khatun *et al.*2001-2002).

Biotechnological approaches for crop improvement is a new research area in Bangladesh. Research on plant biotechnology in bast fiber crops especially on jute has been conducted in few laboratories of Bangladesh and in some other

countries like India, China, and in few laboratories in England. Some achievements have been made in the laboratories of Bangladesh and India using tissue and cell culture techniques. Jute is susceptible to root-knot nematodes and spiral borers. It is also susceptible to stem rot and leaf mosaic diseases. Genes are available against stem borer, fungus and viral diseases which could be inserted in jute in future through genetic transformation. Before developing a protocol for gene transfer in jute, there is a need to develop an efficient and repeatable plant regeneration system from jute explants. The developed protocol for plant regeneration from jute explants then would be used for insertion of agronomically important genes in jute plants in future. At present, marker genes e.g. kanamycin and GUS genes would be used for jute transformation.

Genetically engineered foreign genes have been successfully transferred into several agriculturally important crop plants including jute by *Agrobacterium*-based transformation (Bajaj, 1989). Recently, plants have been engineered to be resistant to herbicide, viruses and insects. Genetic transformation could be one option for the improvement of jute varieties. Insect resistant *Bt* cotton is a very common example of successfully developed insect resistant cotton. This would definitely reduce the cost of pesticide use for cotton production. A vector system is therefore, needed to be developed for the production of transgenic jute expressing agronomically important traits like jute plants with resistant to insect or fungus, with suitable marker genes.

With this view in mind, the present research work has been undertaken for fulfilling the following objectives:

i To establish an efficient and repeatable plant regeneration system for the explants of *C. olitorious*, particularly from the cotyledones (with attached petioles) so that the established system can be used for application of genetic transformation technique.

ii. To know the effect of $FeSo_4$ on shoot regeneration.

Iii. To optimizes the transfer system for the regenerated plantlets in soil.

iv. To know the optimum P^H levels for shoot regeneration.

According to the objectives the following experiments have been undertaken

Experiment 1. *In vitro* seed germination potentiality of *C. olitorious* varieties.

Experiment 2. *In vitro* callus induction performance of *C. olitorious* varieties.

Experiment 3. Optimization of shoot regeneration from the explants of *C. olitorious* varieties at different concentrations of BAP.

Experiment 4. *In vitro* root induction in the regenerated plantlets of *C. olitorious* varieties.

Experiment 5. Effect of different concentrations of $FeSO_4$ on shoot regeneration in *C. olitorious* varieties

Experiment 6. Effects of different levels of p^H on shoot regeneration in *C. olitorious* varieties.

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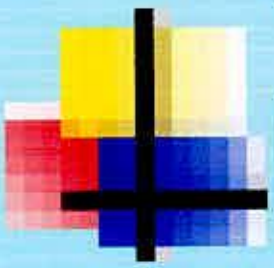


REVIEW OF LITERATURE

In Bangladesh, Jute is the most important fiber crop. Considering that, much attention is given by a large number of researchers on various aspects of production, utilization and improvement. Improvement of jute like other crop through conventional method requires long time. Plant biotechnology, now a day recommends many opportunities for breeders with chances to solve certain breeding problems at cellular level. Biotechnological research on jute has been initiated in early sixties (Islam, 1964). However, output in this relation is still very inadequate.

Modern advances in tissue culture and recombinant DNA technology have opened new opportunity in transformation and improvement of higher plants, which accordingly produced many transgenic plants with new genetic properties. Establishment of an efficient plant regeneration system from the jute explants is a prerequisite to create variability and to introduce foreign genes into this crop through genetic transformation (Khatun, 1993). Nevertheless, some of the important and informative works and findings so far been done to home and abroad on plant regeneration and transformation of jute and other allied fiber crops have been reviewed in this chapter.

2.1 *In vitro* regeneration potentiality of some *Corchorus* genotypes

2.1.1 Concept of tissue culture

Conventional techniques for crop improvement are prolonged processes and take more time for crop improvement. The techniques of plant tissue culture have been developed as a new and powerful tool for crop improvement

(Carlson 1975, Razdan and Cocking 1981) and received wide attention of modern scientists (D'Aamato1978, Skirvin 1978). Regeneration from different explants (leaf, stem, cotyledon, hypocotyls etc.) on different nutrient media under sterile conditions is the basis of plant tissue culture. When explants of a plant are grown in a defined medium, an undifferentiated collection of cells arise which then developed into whole plants from this process is known as regeneration.

Nowadays, plant tissue culture techniques have been come forward as a world wide accepted concept (Hoque, 2001) and opened up several new avenues for manipulation of crop plants to induce genetic changes and selection of desirable traits (Nath, 2001). Besides, plant regeneration from *in vitro* cultures is a prerequisite of genetic transformation techniques in many crop plants (Akter, 2001).

2.1.2 Tissue culture of jute

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

In vitro regeneration has been quite difficult among the species *Corchorus* through tissue culture techniques. It appears that jute is a notorious recalcitrant plant and regeneration is sporadic. Regeneration has only been reported from meristematic tissue but not from totally differentiated tissue, like callus. Where there are reports of regeneration from cotyledon or hypocotyl derived callus, there are usually portions of meristematic tissue left from where regeneration actually occurs.

2.1.3 Need for tissue culture in jute

Cell and tissue culture provides the opportunities for the genetic engineering of this crop to supplement conventional breeding. In immature embryo culture in interspecific hybridization of flax and obtained the interspecific hybrid plant. This is the first study on tissue in bast fiber crops as well as one of the earliest successes in plant tissue culture.

Islam (1981) obtained callus initiation and root formation from explants of *C. olitorius* and claimed to have obtained a few shoots directly from leaf explants of *C. olitorius* on MS medium. However, there was no further report of repeatation of this technique. Plant regeneration of jute from meristem (Rahman *et al.*, 1985), cotyledon (Rahman *et al.*, 1985; Khatun *et al.*, 1992; Ali, 1992), leaf (Islam, 1981), plumule (Das *et al.*, 1986), hypocotyl (Khatun *et al.*, 1992; Ghosh and Chatterjee, 1990; Seraj, 1992), apical meriatems (Khatun, 1993) and anther culture (Islam, 1981) have been reported.

Tissue culture research in jute was started in 1964, when Islam cultured inter specific hybrid embryo (*C. capsularis* × *C. olitorius*) and hybrid plants were obtained. *In vitro* regeneration has been reported to be quite difficult among the species of *Corchorus* through tissue culture technique (Khatun, 1993).

2.1.4 *In vitro* seed germination potentiality of *Corchorus* varieties

2.1. 4.1 *In vitro* seed germination

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

Khatun (1993) conceded out an experiment to study the germination percentage of different varieties of *C.olitorius* namely O-9897, OM-1, O-4, O-72 and CVL-1, CVE-3, D-154 and Tricap-2 of *C. capsularis* on hormone free agar-solidified MS basal media and cotton based MS liquid media. She observed that the germination percentage of the varieties was found higher on cotton based medium than the agar medium.

Khatun (2003) also renowned that the highest germination percentage was found in the variety Tricap-2 (98.66%) on cotton based medium and the lowest germination percentage was originate in the variety O-4 (38.66%) on agar medium.

2.1.5 *In vitro* callus induction performance of *Corchorus* varieties

2.1.5.1 Callus induction

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

2.1.5.2 Varietal difference

Murashige and Skoog (1962) reported that nutritional requirements for optimal growth of tissue *in vitro* may vary with varieties. Even tissue culture of different parts of plant may show different requirements for satisfactory growth.

Saha *et al.* (1999) observed that JRC-312 showed the best shoot regenerative ability followed by JRC-212 and D-154.

Khatun (2003) conducted an experiment on six varieties of jute (CVI-1, CVE-3, D-154, CC-45, BJC and Tricap-2) and observed that the frequency of shoot production varied greatly among the varieties. She reported that Tricap-2 showed best performance in shoot regeneration.

Two species of *Corchorus* was tested for plant regeneration (Khatun, 1993) and she observed that plant regeneration from the explants of *C. olitorius* was very different in culture condition than *C. capsularis*. She have been made several attempts by using various hormone and media combinations *in vitro* to obtain plant regeneration from various explants sources of *C. olitorius*.

2.1.5.3 Effect of explants

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

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

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

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

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

Tewari *et al.* (1999) reported that 2, 4-D induced callusing in 100% of explants when cotyledons, segments of hypocotyls and roots of white Jute (*C. capsularis*) were culturred on MS medium supplemented with 16 adjuvant individually and/or in combination.

Plant regeneration from the explants (cotyledon segments, hypocotyl segments and root segments) of *C. olitorius* (vars. O-9898, OM-1, O-72 and O-4) was difficult. However the explants of cotyledonary petioles of all these varieties produced shoots from the cut ends (Khatun, 2003).

2.1.5.4 Maintenance of callus

Very little work and attention has been paid so far on maintenance of callus of jute. Therefore, maintenance of jute and other crops have been reviewed.

The organogenic callus of *C. capsularis* (vars. D-154 and CVL-1) when rich in large starch granules, was transferred to MS basal medium, and it differentiated into single or multiple shoots (Seraj *et al.*, 1992).

A long-term regeneration system for garlic clones was developed by Myers and Simon (1999) where callus was initiated on modified Gamborg's B-5 medium supplemented with 4.5 µM 2, 4-D and maintained on the same basal medium with 4.7 µM piclorum and 0.4 µM 2ip (isopentenyladenine). Regeneration potential of callus after 5, 12 and 16 months on maintenance medium was measured using several plant growth regulator treatment.

2.1.5.5 Somatic embryogenesis

Somatic embryogenesis has a tremendous potential for large scale production of plant material (Amirato and Styer, 1985) and is considered as an effective aid to genetic transformation study. It represents an alternative for massive clonal propagation and appears to be a potential solution to the problem of field propagation, especially in areas with frequent disease transmission and maintenance of cultivars that have been selected for their important genetic characteristics (De Garcia and Martinez, 1995). There have been very few reports of somatic embryogenesis in jute.

Wang *et al.* (1992) reported that basic MS medium with B5 vitamins and different concentration of Br (brassinolied) at 0.01 ppm + 2, 4-D at 5 ppm induced callus formation in all cultivars except Coker 201 and Coker 312 when he cultured the hypocotyl explants from 8 cotton cultivars. He also reported that after removal of 2, 4-D embryogenesis calluses and embryoids were formed.

Somatic embryogenesis was induced from cotyledon and hypocotyls derived calli, transgenic hairy roots derived callus and protoplast derived callus of *C. capsularis* in the presence of 2, 4-D and BAP (Khatun, *et al.* 1993).

2.1.5.6 Organogenesis

The totipotency of somatic cells has been explained in vegetative propagation of plant species. *In vitro* studies have revealed that most plants would differentiate shoot ends and roots from somatic as well as reproductive tissues. Whole plant regeneration from cultured cells may occur either through shoot-end differentiation of plant from callus has been reported by different workers. The literatures closely related to *in vitro* regeneration of jute are cited below:

Das *et al.* (1986) showed that the plumules of var. D-154 of *C. capsularis* were cultured on BAP and tyrosine fortified MS media, tissue developed into multiple shoots.

Khatun (2001) reported that the cotyledonary explants of *C. olitorius* produced multiple shoots when cultured in MS medium with 0.5 mg IAA/L and 3 mg BAP/L. She also reported that the best *in vitro* response for shoot regeneration was obtained from 0-9897 when she used three varieties of *C. olitorius* (var. 0-4, 0-9897, OM-1).

Khatun (2003) observed multiple shoots from cotyledons with attached petioles explants of *C. capsularis* on MS medium supplemented with 2 mg BAP and 0.5 mg IAA/L.

2.1.5.7 Induction of root

In vitro root induction of jute was reported by several researchers. The information, which are closely related to root induction are reviewed here:

Bigaria (1988) conducted a field experiment to study the influence of IBA, environmental factors and planting position on the regeneration of stem cutting and leaves of *Hibiscus cannabinus*. Stem cutting and leaves were treated with IBA at 10, 25, 50, 100, 150 and 200 ppm. 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 + 0.3 mg IBA/L.

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

Khatun *et al*, (2001-2002) reported that the regenerated plantlets of *C. olitorius* and *C. capsularis* were rooted on MS medium without hormone within seven days.

2.1.6 Optimization of shoot regeneration from the explants of *C. olitorius* varieties at different concentrations of BAP

2.1.6.1 Callus induction

The effect of combination of growth regulators on callus induction from different explants of various jute (*C. capsularis*) varieties were reported by several workers. The most applicable literatures related to callus induction have been reviewed here.

2.1.6.2 Effects of growth regulators

Hypocotyls and cotyledons of 15 upland cultivars (*Gossypium hirsutum*) were cultured in MS medium supplemented with various phytohormones at 16 hour days at 1500-2000 lux. Calluses were formed at 6-10 days, with hypocotyls forming calluses 3-4 days earliar than cotyledons. Morphological traits of the calluses depended on the cultivar and the hormones added to the medium Grey or yellowish-grey friable calluses cultured for 50-60 days produced embryos. while green, white or green and white dense calluses did not. Calluses of cv. ASJ2 on medium supplemented with 0.1 mg kinetin per liter multiplied most rapidly, producing almost double the weight of callus of the other cultivars. but ASJ2 produced the fewest calluses. Zhongmian 12 produced the most calluses, followed by Zhong 13, Stoneville 213 and Coker 312 (Li *et al*., 1989).

Tewari *et al.* (1999) reported that MS medium supplemented with 2, 4-D induced callusing in 100% explants of jute.

Khatun (2003) cultured *in vitro* grown cotyledons (with attached petioles) of *C. capsularis* in agar solidified MS medium supplemented by 0.5 mg/L IAA and different concentration of BAP (2, 3, 4 or 5 mg/L) and she observed performance in callus induction and shoot regeneration on the combination of MS + 0.5 mg/L IAA and 2 mg/L BAP.

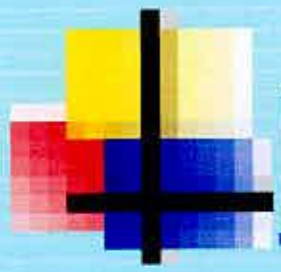
2.1.6.3 Shoot regeneration

Khatun (2003) cultured *in vitro* grown cotyledons (with attached petioles) of *C. capsularis* in agar solidified MS medium supplemented by 0.5 mg/L IAA and different concentration of BAP (2, 3, 4 or 5 mg/L) and she observed best performance in shoot regeneration on the combination of MS + 0.5 mg/L IAA and 2 mg/L BAP.

2.1.7 Effect of different levels of pH on shoot regeneration from cotyledons of *Corchorus* genotypes.

Two scientists of BJRI conducted an experiment on two varieties of *C. capsularis* (Vars. CVL1 and D154) in the presence of a range of pH levels (eg. 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) in association with MS plant regeneration medium. They found that D154 responded for maximum number of shoot regeneration at pH 5.0 which was 65.00% and CVL1 at pH 7.0 which was 63.33%. They also reported that percent shoot regeneration of D154 gradually decreased as pH levels were increased and shoot regeneration of CVL1 gradually increased as the pH levels were increased.

CHAPTER-3



MATERIALS AND METHODS

3.1 Experimental Materials

Explants (cotyledon with attached petioles) of *Corchorus olitorious* such as O-9897, O-72, O-4 and OM-1 were used in the present investigation.

3.2 Sources of the experimental materials

The experimental materials used in this trial were collected from of Bangladesh Jute Research Institute (BJRI), Dhaka.

3.3 Location, time, duration and year

The experiments were conducted in the Genetic Engineering Laboratory, Cytogenetics Department, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from March 2005 to May 2006.

3.4 Media used

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

A. For seed germination

MS (Murashige and Skoog, 1962) basal medium supported by with clinical cotton or agar.

B. For callus induction and shoot differentiation

i. MS (Murashige and Skoog, 1962) medium without hormone supplement.

ii. MS medium supplement with IAA 0.5 ml/L and 2 mg/L BAP

C. For root initiation

i. MSO medium (hormone free MS medium).

3.5 Methods

3.5.1 Preparation of culture media

In this trial, for the induction of callus and plantlet regeneration in jute a number of culture media have been prepared and advocated of which MS (Murashige & Skoog, 1962) medium was used for the present research work. A nutrient medium consists of organic and inorganic salts, irons, a carbon source, some vitamins and growth regulators were used. Composition of MS medium formulated by Murashige & Skoog, 1962, is presented in Appendix I.

Different steps of media preparation are described:

3.5.2 Preparation of stock solutions

Stock solutions of growth regulators were prepared separately by dissolving the desired quantity of ingredients in appropriate solvent and the required final volume was made with water for ready use. Separate stock solutions for macronutrients, micronutrients, irons, vitamins, growth regulators etc. were prepared and stored appropriately for use.

i) Stock solution A (macronutrients)

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

ii) Stock solution B (micro-nutrients)

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

iii) 0.028 gm ferrous sulphate powder was directly added to the medium.

iv) Stock solution D (Vitamins)

Each of the desired ingredients except myo-inositol were taken at 100 folds (100×) of their final strength in a measuring cylinder and dissolved in 75 ml of distilled water. Then the final volume was made up to 100 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots and stored at -20°C. Myo-inositol was used directly at the time of media preparation.

v) Hormonal stock solutions

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

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

A. Auxin: 3-indole acetic acid (IAA)

B. Cytokinins: 6-benzyl amino purine (BAP)

The growth regulators were dissolved in appropriate solvent as IAA in ethanol and BAP in 0.1N NaOH.

For the preparation of stock solution of any of these hormones, 10mg of each of the hormone powder was taken on a clean beaker and dissolved in 1 ml of the particular solvent. The mixture was then collected in a 20 ml measuring cylinder and volume was made up to 10 ml by the further addition of distilled water. The solution was then poured into a clean glass container and stored at 4°C and used for maximum period of two weeks.

3.5.3 Steps followed for the preparation of culture media

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

3.5.4 Preparation of MS medium

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

i) 100 ml of macronutrients, 10 ml of micronutrients, .028 gm iron powder and 10 ml of vitamins were taken from each of these stock solutions into a 2 litre Erlenmeyer flask on a heater cum magnetic stirrer.

ii) 500 ml distilled water was added in the flask to dissolve all ingredients.

iii) 100 mg of myo-inositol was added directly to the solution and dissolved.

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

v) Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

vi) The whole mixture was then made up to 1000 ml .

vii) pH of the medium was adjusted to 5.8 with a digital pH meter with the help of 0.IN NaOH or 0.IN HCl, whichever was necessary.

viii) After adjustment the pH, 9 g agar was added to solidify the medium. The mixture was then heated gently with continuous stirring till complete dissolution of agar.

ix) Required volume of hot medium was dispensed into culture vessels or conical flasks. After dispensing the medium the culture vessels were plugged with cork and/or non-absorbent cotton and marked with different codes with the help of a glass marker to indicate specific hormonal combinations.

3.6 Sterilization

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

3.6.1 Sterilization of culture media

The conical flasks containing prepared media were autoclaved at 1.16 $kgcm^{-2}$ pressure and 121^0C temperature for 22 minutes. The LB medium, was autoclaved for 15 minutes at 1.16 kg/cm^2 pressure and 121^0C temperature. The medium was then poured into sterile petri dishes and sterile culture vessels (flasks) in a laminar air flow cabinet and were allowed to be cool before use. All the petri dishes and vials were marked with permanent marker or sticker to indicate specific phytohormonal combinations.

3.6.2. Sterilization of glassware and instruments

Beakers, test tubes, conical flasks, pipettes, metallic instruments like forceps, scalpels, inoculation loops micropipette tips, eppendorf tubes, needles, spatulas were wrapped with aluminum foils, vials were capped with plastic

cap and then were sterilized in an autoclave at a temperature of 121 °C for 30 minutes at 1.16 $kgcm^{-2}$ pressure.

3.6.3 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.

3.6.4 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 absolute ethyl alcohol to overcome the surface contaminants. During the entire period of inoculation the autoclaved scalpels, forceps and inoculation loop were kept immersed into absolute alcohol contained in a glass jar inside the cabinet. At the time of inoculation these were again sterilized by flaming method inside the cabinet. Both the hands were rinsed with 70% alcohol. All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants.

3.7 Culture techniques:

3.7.1 Development of a protocol for *in vitro* seed germination of *C. olitorious* varieties on agar-based and clinical cotton supported medium

Seeds of *C. olitorious* (variety O-9897, O-72, O-4 and OM-1) were surface sterilized by immersion in 0.1% (w/v) Mercuric Chloride for 20 minutes, followed by 6 washes with sterile deionized water. Seeds are germinated on the surface of 50ml aliquots of hormone free agar-solidified (0.8%, w/v) MS

basal medium contained in 100 ml capacity of conical flasks. Fifty seeds were inoculated in three flasks.

In another set of experiment, surgical cotton was used instead of agar in association with MS basal medium. Cotton-based liquid medium was used for seed germination. Surgical cotton (3 mg) was placed at the bottom of 100ml flasks. Each flask contained 25ml of hormone free MS liquid medium. Seeds of *C. olitorious* varieties were surface sterilized with 0.1% (w/v) Mercuric Chloride for 20 min and placed on the surface of cotton-based MS medium. Cultures were placed in a growth room with 28^0C under 1.0Wm^{-2} of daylight fluorescent tubes with 12 hour photoperiod. Fifty seeds were inoculated in three flasks. 8-9 days old seedlings were used for data collection.

3.7.2 *In vitro* callus induction performance of some *Corchorus olitorious* varieties

The following culture methods were employed in the present investigation:

a) Explant culture

b) Subculture

a) Explant culture

The seedlings raised in axenic culture were used as the source of explants. The cotyledons with attached petioles were used as explants. Cotyledons with attached petioles of *C. olitorious* were taken from *in vitro* grown seedlings for this study. In this case, seedlings were allowed to develop for 8-9 days to make sure that the undeveloped apical shoot buds were not attached with the petioles. Therefore, the optimum explants source, cotyledons with their attached petioles were excised from 8-9 days old seedlings and were cultured in 250ml conical flasks containing 50 ml of agar-solidified MS medium with

IAA (0.5 mg/l) and BAP (3.0 mg/l). Eight explants were inoculated in each culture flask.

The culture flasks containing explants were placed in a growth room and maintained at 28^0C under 1.0 Wm^{-2} of daylight fluorescent tubes with a 12 hrs photoperiod. The culture flasks were checked daily to note the response of development and contamination. Data were recorded 6 weeks after culture.

b) Subculture or transfer

i) Subculture of the callus for shoot regeneration

Two weeks after inoculation of explants, the calli attained convenient size. Then they were removed aseptically from the cultured flask on a sterilized glass plate inside the laminar airflow cabinet and were placed again on freshly prepared sterilized medium containing no hormone. The culture flasks showing signs of contamination were discarded.

ii) Transfer of regenerated shoot-buds for root induction

When the 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 250ml conical flask with freshly prepared MSO (hormone free MS medium) medium for root production. The conical flasks containing plantlets were incubated at 28^0 C under a 1.0 Wm^{-2} of daylight fluorescent tubes with a 12 hrs photoperiod. Day to day observations was carried out to note the responses.

3.7. 3 Optimization of shoot regeneration from the explants of *Corchorus olitorious* varieties at different concentrations of BAP

The following culture techniques were employed in the present study:

a) Explant culture

b) Subculture

a) Explant culture

The seedling raised in axenic culture was used as the source of explants. Here cotyledons (with attached petioles) were used as explants. Eight explants were inoculated in each culture flask containing different treatments of BAP (1 mg/l, 2 mg/l, 3 mg/l and 4 mg/l) and constant IAA (0.5 mg/l). The culture flasks containing explants were placed under fluorescent light in a growth room with controlled temperature (28^0C).

c) Subculture of the callus for shoot regeneration

Discussed in 3.7.2 (i)

3.7.4 Effect of different concentrations of $FeSo_4$ on shoot regeneration in *Corchorus olitorious*

The following culture techniques were employed in the present study:

a) Explant culture

b) Subculture

a) Explant culture

The seedling raised in axenic culture was used as the source of explants. Here coltyledons (with attached petioles) were used as explants. Eight explants were inoculated in each culture flask containing different Concentration of $FeSO_4$ (0 mg/l, 28 mg/l, 56 mg/l, 84 mg/l and 112 mg/l). The culture flasks

containing explants were placed under fluorescent light in a growth room with controlled temperature (28^0C).

d) Subculture of the callus for shoot regeneration

Discussed in 3.7.2 (i)

3.7. 5 Effect of different levels of pH on shoot regeneration in *Corchorus olitorious*

The following culture techniques were employed in the present study:

a) Explant culture

b) Subculture

a) Explant culture

The seedling raised in axenic culture was used as the source of explants. Here coltyledons (with attached petioles) were used as explants. Ten explants were inoculated in each culture flask containing different levels of pH (4.0, 4.5, 5.0. 5.5, 6.0, 6.5, 7.0 and 7.5) supplemented with 3 mg/l BAP and 0.5 mg/l IAA. The culture flasks containing explants were placed under fluorescent light in room with controlled temperature (28^0C) until callus initiation. The flasks were checked daily to note the appearance of callus.

b) Subculture of the callus for shoot regeneration

Discussed in 3.7.2 (i).

3.8 Recording of data

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

a) Percent seed germination

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

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

b) Per cent callus induction

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

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

c) Percent shoot regeneration

The percentage of plant regeneration was calculated based on the number calli transferred to regeneration medium and the number of calli produced plantlets

$$\text{Percent shoot regeneration} = \frac{\text{Number of calli with plantlet}}{\text{Number of explants incubated}} \times 100$$

d) Days of callus initiation

Generally callus initiation started after six days of incubation of explants. The number of callus initiation over a number of days was recorded. The mean value of the data provided the days required for callus initiation.

e) Days to shoot initiation

Shoot initiation started after 15-21 days of incubation of explants. The number of shoots proliferated over a number of days were recorded. The mean value of data provided the days required for shoot initiation.

f) Average number of shoot per callus

Number of shoot per callus was recorded at 21 days interval and the mean was calculated using the following formula:

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

where,

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

$\sum$ = summation

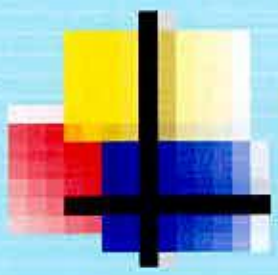
Xi = number of shoots/callus

n = number of observation

3.7 Statistical analysis of data

The data for different components under present trial were statistically analyzed to find out the significance of the difference. The experiments were conducted in Completely Randomized Design (CRD) with three replications. The analysis of variance for different components was performed and means were compared by Least Significant Differences (LSD) at 0.05 level of probability.

CHAPTER-4



RESULTS AND DISCUSSION

4.1 Experiment 1. *In vitro* seed germination potentiality of *C. olitorious* varieties

The experiment was conducted for regeneration of healthy seedlings from jute seeds required for subsequent experiments. In the present experiment seeds of *C. olitorious* (O-9897, OM-1, O-4 and O-72) were germinated on both cotton-based liquid and agar solidified medium. The number of seeds germinated was presented in percent and its interpretations have been given under the following headings:

4.1.1 Effect of variety

Number of seed germination

Significant variation was recorded among the different varieties of *C. olitorious* where 50 seeds were blotting for germination (Table1). The highest number of germinated seeds was counted for the variety O-72 (35.84) which was followed by variety OM-1 (34.08). On the other hand the lowest number of seed germinated in variety O-4 (30.59). Variation of seed germination occurred due of variation in dormancy in the varieties.

Percent seed germination

A statistically significant variation was recorded among the different varieties of *C. olitorious* considering percentage of germinated seed under the present trial (Table 1). The highest percentage of germination was counted for the variety O-72 (71.68%) which was closely followed by variety OM-1 (68.16%) and O-9897 (67.78%). On the contrary the lowest percentage of germination

was counted in variety O-4 (61.17%). Such results may be due to seeds dormancy or may be physiological reason of different varieties.

4.1.2 Effect of media

Number of seed germination

A significant variation was recorded in reflection the number of germinated seed in MS media and the results are presented in (Table 2). The highest number of germinated seeds (45.79) was counted in cotton-based liquid medium and the lowest germinated seeds were recorded in agar solidified medium. From these results it was found that cotton-based liquid media was the more suitable for the germination of *C. olitorious* than agar solidified medium. Similar results also reported earlier by Khatun (2003). This might be due to the more aeration facilities of cotton based liquid media.

Percent seed germination

Statistically significant variation was recorded in consideration the percentage of germination in MS media (Table 2). The highest percentage of germination (91.58%) was counted in cotton-based liquid medium. On the other hand the lowest percentage of seed germination (42.82%) was recorded in agar solidified medium. From these results it was found that cotton-based liquid media was the more suitable for the germination of *C. olitorious* than agar solidified medium.

4.1.3 Combined effect of variety and media

Number of seed germination

The combined effect between variety and culture media also showed significant statistical differences in consideration the number of germinated seeds under the present trail. The highest number of germinated seed (48.78)

was recorded in variety O-72 in cotton-based liquid medium (Table 3). The lowest number of germinated seed (19.51) was counted in variety O-4 with agar solidified medium which was closely followed (21.56) by O-9897 variety with agar solidified media.

Percent seed germination

The combined effect between variety and MS media also showed statistically significant differences in the percentage of germination seeds. The highest percentage of seed germination (97.55%) was recorded in variety O-72 in cotton-based liquid culture medium (Table 3). The lowest percentage of seed germination (39.02%) was recorded in variety O-4 with agar solidified medium which was closely followed by OM-1 variety with agar solidified media.

Seeds of the varieties of *C. olitorius* germinated on both agar and cotton supported MS medium. The varieties ensured 83.33 to 97.55 % seed germination on cotton supported MS liquid medium, whereas, for agar solidified Ms medium, seed germination was as low as 39.02 to 45.80 % for the same varieties. The differences in percentage of seed germinated between agar supported medium and cotton supported liquid MS medium was found to be statistically significant. Similar finding was reported for seed germination of white jute varieties (Naher *et al.*, 2003). Though the result of seed germination of *C. olitorius* varieties was found to be more marked than *C. capsularis*, both of the species responded better in cotton supported liquid MS medium. The seedlings of C. olitorius varieties grown on cotton supported liquid MS medium was found to be much more helthy than the seedlings grown on agar supported MS medium. Similar response was reported for *C.*

capsularis. (Naher *et al.*, 2003). This result could be a valuable addition for tissue culture system as cotton supported seed germination system was comparatively cheaper than agar supported system. Moreover, medium consumption is lesser (i.e.20ml/flask) for cotton supported system, whereas, for agar supported system, medium requirement was 50 ml/flask.

Table 1. Seed germination of different varieties of jute

Variety	No. of seed germinated	Percent seed germination
09897	33.89 b	67.78 b
OM-1	34.08 b	68.16 b
O-4	30.59 c	61.17 c
O-72	35.84 a	71.68 a
Level of Significance	**	**
$LSD_{0.05}$	0.647	1.294

Table 2. Seed germination under different culture media in jute

Culture media	No. of seed germinated	Percent seed germination
Cotton	45.79 a	91.58 a
Agar	21.41 b	42.82 b
Level of Significance	**	**
$LSD_{0.05}$	0.458	0.915

Table 3. Combined effect of variety and culture media on seed germination in jute

Variety × Culture Media		No. of seed germinated	Percent seed germination
09897	Cotton	46.22 b	92.44 b
	Agar	21.56 e	43.12 e
OM-1	Cotton	46.50 b	92.99 b
	Agar	21.66 e	43.33 e
O4	Cotton	41.66 c	83.33 c
	Agar	19.51 f	39.02 f
O72	Cotton	48.78 a	97.55 a
	Agar	22.90 d	45.80 d
Level of Significance		**	**
$LSD_{0.05}$		0.916	1.830

Plate 1. Seed germination on clinical cotton based MS liquid medium

4.2 Experiment-2 *In vitro* callus induction performance of *C. olitorious* varieties

Plant regeneration through callus induction offers unique facilities of reproducible protocol as well as recovery of somaclonal variants which can be utilized for the future crop improvement program. Therefore induction of calli from cotyledonary explants and subsequent regeneration of complete plantlets is very important.

4.2.1 Effect of varieties

Number of explants producing callus

Significant variation was recorded among the varieties of *C. olitorious* for c number of explants producing callus (Table 4). The highest number of explants producing callus were recorded for the variety O-9897 (19.47) which was closely followed by variety O-4 (18.58). On the other hand, the lowest number of explants producing callus were recorded in variety OM-1 (15.93).

Percent callus induction

Significant variation was recorded among the varieties of *C. olitorious* for percent callus induction (Table 4). The highest percent of callus inductions was recorded for the variety O-9897 (54.07%) which was closely followed by variety O-4 (51.62%) and the lowest percent callus induction was counted in variety OM-1 (44.26%).

4.2.2 Effect of different concentration of BAP

Number of explants producing callus

Significant variation was recorded for number of explants growing callus in different concentrations of BAP(Table 5). The highest number of explants producing callus (19.96) was recorded in concentration of in 3 mg/l BAP

which was closely followed (18.51) by 2 mg/l. On the other hand, the lowest number of explants producing callus (16.07) was recorded in BAP concentration of 4 mg/l which was closely followed (17.44) by 1 mg/l (Plate 2).

Percent callus induction

Statistically significant variation was recorded in considering the percent callus induction in different concentration of BAP (Table 5). The highest percent callus induction (55.43%) was recorded in concentration of BAP 3 mg/l which was closely followed (51.42%) by concentration of BAP 2 mg/l. On the other hand the lowest percent callus induction (44.63%) was recorded in concentration of BAP 4 mg/l which was closely followed (48.45%) by concentration of BAP 1 mg/l.

4.2.3 Combined effect of varieties and different concentration of BAP

Number of explants producing callus

The combined effect between variety and concentration of BAP also showed a statistically significant difference in respect of number of explants producing callus under the present experiment (Table 6). The highest number of explants producing callus (21.60) was recorded in variety O-9897 with concentration of BAP 3 mg/l which was closely followed by variety O-4 with concentration of BAP 3 mg/l (20.33) and O-9897 with BAP concentration 2 mg/l. The lowest number of explants producing callus (14.00) was recorded in variety OM-1 with concentration of BAP 4 mg/l which was closely followed by OM-1(15.57) with BAP concentration 1 mg/l.

Percent callus induction

The combined effect between variety and concentration of BAP also showed a statistically significant difference in respect of percent callus induction under the present piece of experiment (Table 6). The highest percent callus induction (60.00%) was recorded in variety O-9897 with concentration of BAP 3 mg/l which was closely followed (56.48%) with variety O-4 with concentration of BAP 3 mg/l and variety O-9898 with BAP concentration 2 mg/l. The lowest average percent callus induction (38.89%) was counted in variety OM-1 with concentration of BAP 4 mg/l which was closely followed (43.24%) by variety OM-1 with concentration of BAP 1 mg/l.

Table 4. Effect of varieties of *C. olitorious* on callus induction

Variety	No. of explants producing callus	Percent callus induction
O-9897	19.47 a	54.07 a
OM-1	15.93 c	44.26 c
O-4	18.58 b	51.62 b
Level of significance	**	**
LSD	0.476	1.321

Table 5. Effect of different concentrations of BAP on callus induction

Concentration of BAP (mg/l)	No. of explants producing callus	Percent callus induction
1	17.44 c	48.45 c
2	18.51 b	51.42 b
3	19.96 a	55.43 a
4	16.07 d	44.63 d
Level of significance	**	**
LSD	0.550	1.526

Table 6. Combined effect of varieties of *C. olitorious* and concentrations of BAP on callus induction

Variety × Concentration of BAP (mg/l)		No. of explants producing callus	Percent callus induction
O-9897	1	19.00 c	52.77 c
	2	20.20 b	56.11 b
	3	21.60 a	60.00 a
	4	17.07 de	47.40 de
OM-1	1	15.57 f	43.24 f
	2	16.23 ef	45.09 ef
	3	17.93 d	49.82 d
	4	14.00 g	38.89 g
O-4	1	17.77 d	49.35 d
	2	19.10 c	53.06 c
	3	20.33 b	56.48 b
	4	17.13 de	47.59 de
Level of Significance		NS	NS
LSD		--	--

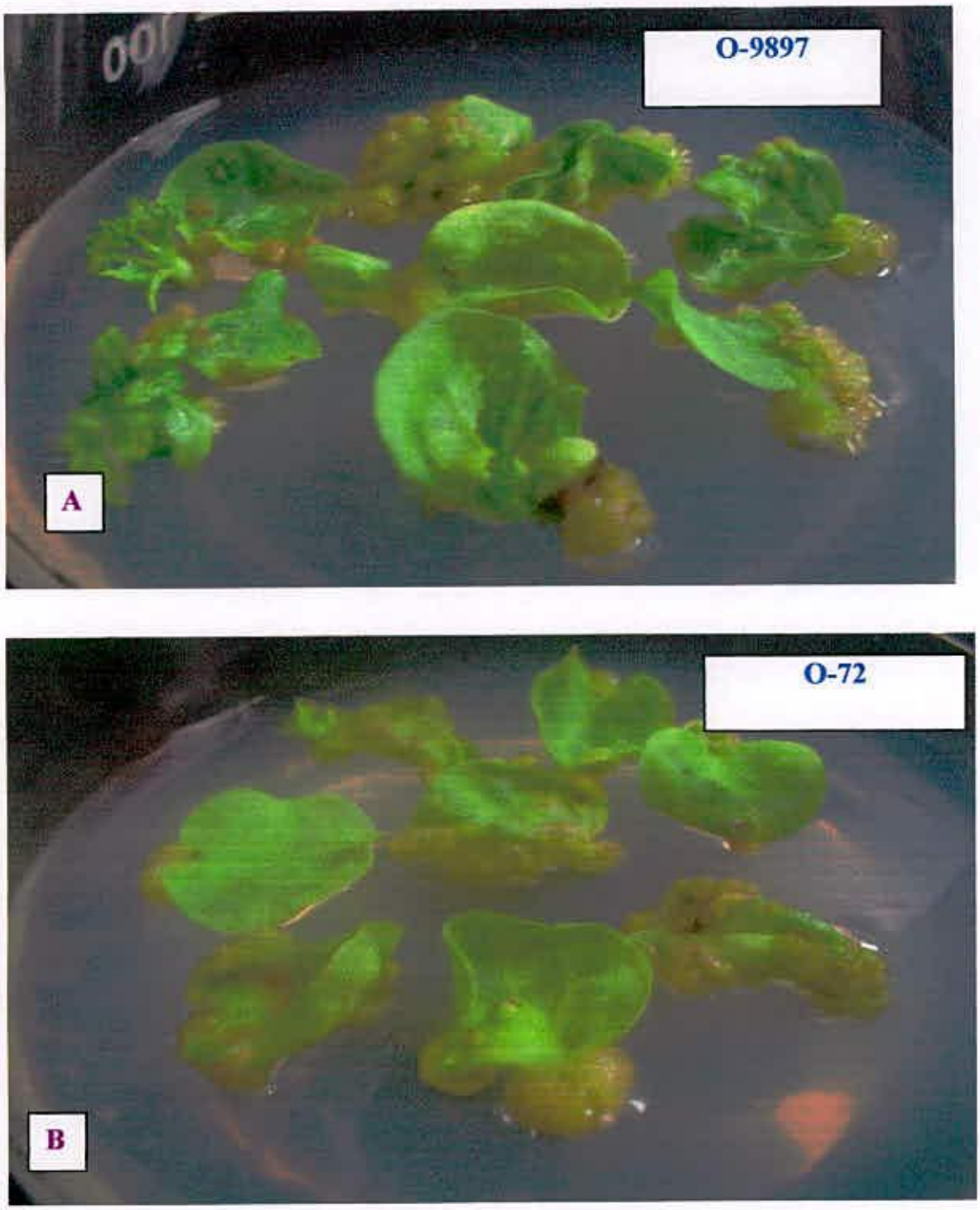


Plate 2. Callus initiation of O-9897 and O-72 on MS+0.5 mg/l IAA + 3 mg/l BAP (A: O-9897 and B: O-72)

4.3 Experiment - 3: Optimization of shoot regeneration from the explants of *C. olitorious* varieties at different BAP concentrations

In this experiment different concentration of BAP and constant IAA were used for shoot regeneration using cotyledonary petiole as explants to know optimum concentration of BAP for plant regeneration.

4.3.1 Effect of varieties

Average number of cotyledon regenerated

Significant variation was recorded among the different varieties of *C. olitorious* on consideration of average number of cotyledon regenerated (Table7). The highest average numbers of cotyledons regenerated was recorded for the variety O-72 (53.66) which was closely followed by variety O-9897 (51.05). On the other hand, the lowest number of cotyledon regenerated was counted in variety OM-1 (48.61).

Percentage of cotyledon producing shoots

Significant variation was recorded among the different varieties of *C. olitorious* on consideration of percentage cotyledon producing shoots (Table 7).The highest percentage cotyledons producing shoots was recorded for the variety O-72 (53.87%) which was closely followed by variety O-9897 (51.25%). On the other hand the lowest percentage cotyledon producing shoots was counted in variety OM-1 (46.02%).

Average number of shoots produced by each cotyledon

A statistically significant variation was recorded among different varieties of *C. olitorious* on consideration of average number of shoots produced by each cotyledon under the present piece of experiment in laboratory condition (Table 7). The highest average numbers of shoots produced by each cotyledon was

recorded for the variety O-9897 (6.79) which was closely followed by variety O-72 (6.40). On the other hand, the lowest average numbers of shoots produced by each cotyledon was recorded in variety OM-1 (4.71).

4.3.2 Effect of different concentration of BAP

Average number of cotyledon regenerated

Considering the average number of cotyledons regenerated, a statistically significant variation was found in different concentrations of BAP (Table 8).The highest number of cotyledon regenerated (57.83) were recorded in concentration of BAP 3 mg/l. On the other hand the lowest numbers (45.33) were recorded in BAP concentration 1 mg/l (Plate 3).

Percentage of cotyledon producing shoots

A statistically significant variation was recorded in considering the percentage cotyledon producing shoots in different concentration of BAP (Table 8). The highest percentage cotyledon producing shoots (56.19%) were recorded in BAP concentration 3 mg/l which was closely followed (50.98%) by BAP concentration 2 mg/l. On the other hand the lowest percentage cotyledon producing shoots (45.66%) were recorded in concentration of BAP 1 mg/l which was closely followed by BAP concentration 4 mg/l.

Average number of shoots produced by each cotyledon

In consideration of the average number of shoots produced by each cotyledon. a statistically significant variation was found in different concentration of BAP (Table 8). The highest average numbers of shoots produced by each cotyledon (6.18) were recorded in BAP concentration 3 mg/l which was statistically identical with concentration 2 mg/l and 4 mg/l. On the other hand, the lowest

average numbers of shoots produced by each cotyledon (5.39) were recorded in BAP concentration 1 mg/l.

4.3.3 Combined effect of varieties and concentration of BAP

Average number of cotyledon regenerated

The combined effect between variety and concentration of BAP also showed statistically significant differences in respect of average number of cotyledon regenerated under the present piece of experiment. The highest number of cotyledon regenerated (59.68) was recorded in variety O-72 with concentration of BAP 3 mg/l which was statistically identical with variety O-9897 with concentration of BAP 3 mg/l. The lowest average number of cotyledon regenerated (41.60) was counted in variety OM-1 with concentration of BAP 1 mg/l. The details results are presented in Table 9.

Percentage of cotyledon producing shoots

The combined effect between varieties and concentration of BAP also demonstrated statistically significant differences in respect of percentage cotyledon producing shoots under the present trial. The highest percentage cotyledon producing shoots (59.54%) was recorded in variety O-72 with concentration of BAP 3 mg/l which was statistically similar with variety O-9897 with concentration of BAP 3 mg/l (Table 9). The lowest average percentage cotyledon producing shoots (42.60%) was counted in variety OM-1 with concentration of BAP 1 mg/l.

Average number of shoots produced by each cotyledon

Combined effect between varieties and concentration of BAP also showed statistically significant differences in respect of average number of shoots produced by each cotyledon under the present trial in laboratory condition.

The highest average number of shoots produced by each cotyledon (7.40) was recorded in variety O-72 with concentration of BAP 4 mg/l which was statistically matching (7.29 & 7.00) with variety O-9897 with concentration of BAP 2 & 3 mg/l (Table 9). The lowest average number of shoots produced by each cotyledon (4.03) was counted in variety OM-1 with concentration of BAP 1 mg/l.

Shoot regeneration was observed from the cotyledons of *C. olitorius* in the presence of high level of BAP (3.0 mg/l). This result is comparable with shoot regeneration from cotyledons of other fiber producing plants like kenaf, where, plant regeneration was obtained with BAP (3.0 mg/l and 5.0 mg/l) and from *C. capsularis* BAP (2.0 v mg/l). Two species of jute (*C. capsularis* and *C. olitorius*) and kenaf responded similarly hormone combination like IAA and BAP.

In this study, differences were noticed among the varieties of *C. olitorius* in percentage of cotyledons producing shoots and also in number of shoots produced by contyledon^{-1}. Similar observation was reported earlier by Khatun *et al.* (1993) and Naher *et.al.* (2003) for plant regeneration from 10 accessions of *C. capsularis* cotyledons. Naher *et. al.* (2003) demonstrated that the differences were noticed among the 10 accessions of *C. camsularis* in percentage of cotyledons producing shoots and also in number of shoots produced by cotyledon^{-1}. All these reports were in agreement with the results of the present investigation on jute for varietals differences.

Plant regeneration from different explants of *C. olitorius* and *C. capsularis* have been tried and reported by Khatun (1993). A detail study of cotyledon

segments, hypocotyls segments and root segments have been conducted for morphogenic responses of jute by using various combinations and concentrations of auxins and cytokinins. Plant regeneration was not obtained from any explants including the cotyledons without petioles attached. The present finding also indicates that the cotyledons of *C. olitorius* will respond to plant regeneration provided the petioles remain attached and that makes tossa jute (*C. olitorius*) comparable to apple (Kouider *et. al.* 1984); *Brassica* spp. (sharma *et al.*, 1991); white jute *C. capsularis* (Khatun *et al.* 1993) and kenaf (Khatun *et. al.* 2003). All these species similarly required an attached petioles for their cotyledons to undergo morphogenesis. Like wise, cultured leaves of *Echeveria elegans* require an attached petiole for plant regeneration (Raju and Mann, 1970).

Table 7. Percentage of shoot regeneration from the cotyledons (with attached petioles) of different varieties of *C. olitorious*

Variety	Average number of cotyledon regenerated	% Cotyledons producing shoots	Average number of shoots produced by each cotyledon
O-9897	51.05 b	51.25 b	6.79 a
O72	53.66 a	53.87 a	6.40 b
OM-1	48.61 c	46.02 c	4.71 c
Level of Significance	**	**	
$LSD_{0.05}$	0.895	0.957	0.198

Table 8. Percentage of shoot regeneration from the cotyledons (with attached petioles) of different concentration of BAP

Concentration of BAP (mg/l)	Average number of cotyledon regenerated	% Cotyledons producing shoots	Average number of shoots produced by each cotyledon
1	45.33 c	45.66 d	5.39 b
2	51.12 b	50.98 b	6.13 a
3	57.83 a	56.19 a	6.18 a
4	50.14 b	48.69 c	6.17 a
Level of Significance	**	**	**
$LSD_{0.05}$	1.033	1.105	0.228

Table 9. Percentage of shoot regeneration from the cotyledons (with attached petioles) of different varieties of *C. olitorious* using MS basal medium supplemented by BAP

Variety × Concentration of BAP (mg/l)		Average number of cotyledon regenerated	% Cotyledons producing shoots	Average number of shoots produced by each cotyledon
O-9897	1	45.23 f	45.23 f	6.57 b
	2	53.23 bc	53.23 b	7.00 a
	3	59.14 a	59.22 a	7.29 a
	4	46.60 f	47.33 de	6.30 b
O72	1	49.17 e	49.17 cd	5.57 c
	2	53.20 bc	53.20 b	6.40 b
	3	59.68 a	59.54 a	6.24 b
	4	52.60 cd	53.57 b	7.40 a
OM-1	1	41.60 g	42.60 g	4.03 e
	2	46.93 f	46.50 ef	5.00 d
	3	54.67 b	49.80 c	5.00 d
	4	51.23 d	45.17 f	4.80 d
Level of Significance		**	**	**
$LSD_{0.05}$		1.789	1.914	0.395

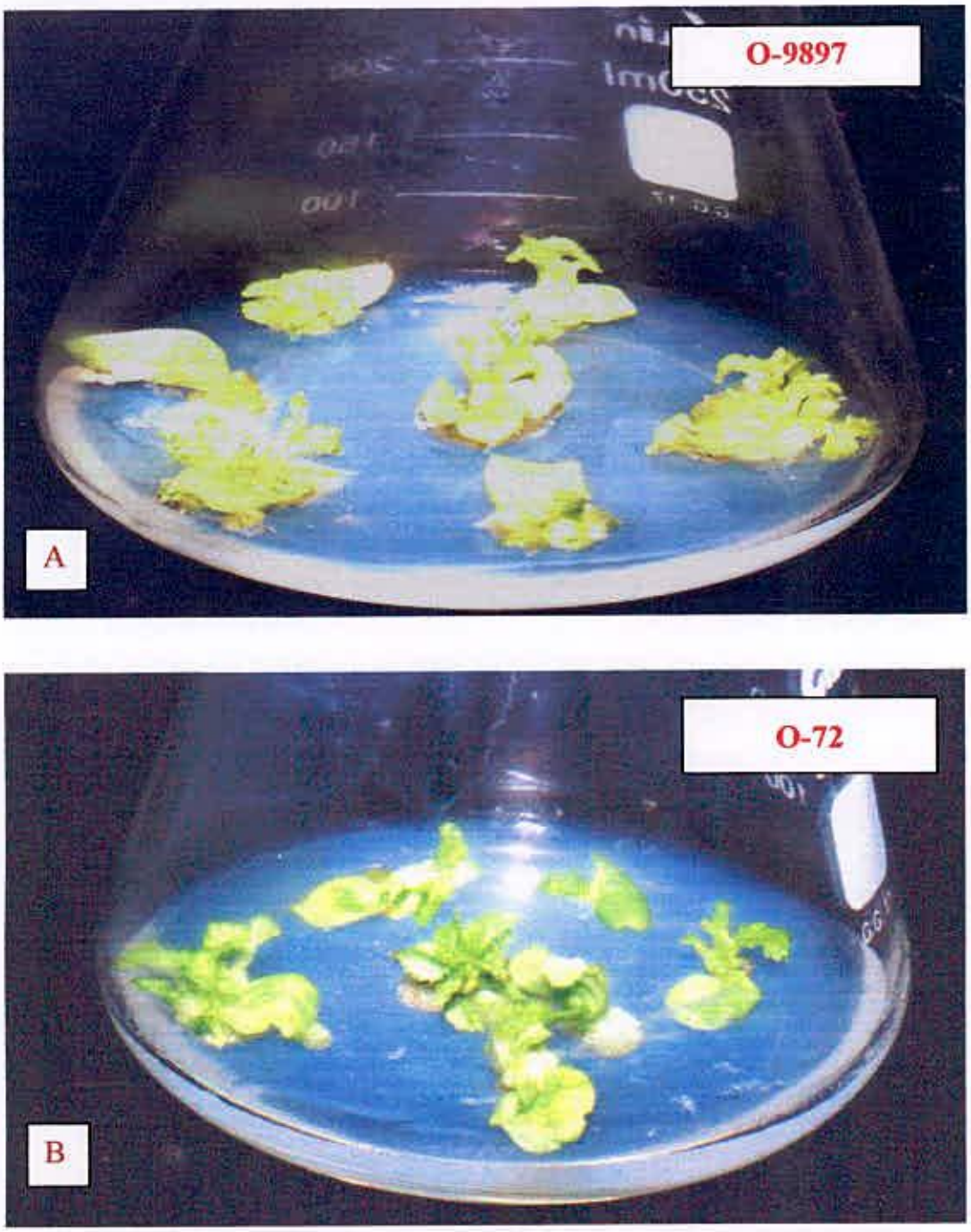


Plate 3. Shoot regeneration from O-9897 and O-72 on MS+0.5 mg/l IAA + 3 mg/l BAP (A: O-9897 and B: O-72)

4.4 Experiment-4 *In vitro* root induction in the regenerated plantlets of *C. olitorious* varieties

For *In vitro* root induction from the regeneration plantlets variety O-9897, O-72 and OM-1 were used with hormone free MS media.

Number of shoot to which root induced

A statistically significant variation was recorded among the different varieties of *C. olitorious* on consideration of number of shoots to which root induced under the present trial in laboratory condition. The highest numbers of shoots to which root induced were recorded for the variety O-9897 (19.30) which was statistically similar by variety O-72 (17.80). On the other hand, the lowest numbers of shoot to which root induced were recorded in variety OM-1 (14.50). The results were presented in Table 10.

Percentage of root initiation

Considering percentage root initiation a statistically significant variation was recorded among the different varieties of *C. olitorious* under the present piece of experiment in laboratory condition (Table 10). The highest percentage of root initiations was recorded for the variety O-9897 (53.61%) which was statistically identical by variety O-72 (49.44%). On the contrary the lowest percentage root initiations were recorded in variety OM-1 (40.28%) (Plate 4).

Days to root initiation

A statistically significant variation was recorded in consideration days to root initiation among the different varieties of *C. olitorious* under the present trial in laboratory condition. The highest days to root initiation were required for the variety OM-1 (10.40 days) which was closely followed by variety O-72

(9.50 days). On the other hand, the lowest days to root initiation were required in variety O-9897 (8.20 days). The results are presented in Table 10.

Root production was readily obtained from different explants and callus of tossa jute *C. olitorius* and similar result was obtained from cotyledons of white jute (*C. capsularis*) (Khatun, 1993; Nahar *et al.*, 2003). Recently, vigorous root production was reported from the cotyledonary petioles of kenaf (*Hibiscus cannabinus* var. HC-2) at various concentrations of auxins and cytokinins used (Khatun *et al.*, 2003). The present finding also shows similarity with the findings of Srivatanakul *et al.*, (2000). They reported that rooting of regenerated kenaf shoots was not found to be difficult to achieve, even, with the presence of high levels of TDZ, which usually act as a potent cytokinin. They have reported that TDZ inhibited root production in woody plant tissue culture but not inhibited root production for kenaf explant culture. Root production was also reported in mesta (Khatun *et al.*, 2001-2002). All of these species e.g. jute, kenaf and mesta are fibre producing plants. From this result, it may be concluded that there might be a relationship between root production and fibre producing plants. These species might contain high level of auxins that are favorable for root production.

Table 10. Effect of different varieties on number of shoots to which roots induced, percentage of root initiation and days to root initiation

Variety	No. of shoot to which root induced	Percentage of root initiation	Days to root initiation
O-9897	19.30 a	53.61 a	8.20 c
O-72	17.80 b	49.44 a	9.50 b
OM-1	14.50 c	40.28 b	10.40 a
Level of Significance	**	**	**
LSD	1.834	5.096	0.774

Experiment 5. Effect of different concentration of $FeSO_4$ on shoot regeneration in *C. olitorious* varieties

To identify the optimum concentration of $FeSO_4$ for regeneration of *C. olitorious* the cotyledon were cultured as explants in this experiment.

4.5.1 Main effect of varieties

Number of explants showing shoots

No significant variation was recorded among the different varieties of *C. olitorious* on consideration of number of explants showing shoots under the present piece of experiment in laboratory condition. However, the highest number of explants showing shoot (5.47) regeneration was recorded for the variety O-9897 and the lowest were counted in variety O-72 (5.33). The results are presented in Table 11.

Percent shoot regeneration

No significant variation was recorded among the different varieties of *C. olitorious* on consideration of percent shoot regeneration under the present piece of experiment. (Table 11) but the highest number of percent shoot regeneration (45.55%) were recorded for the variety O-9897 and the lowest percent shoot regeneration were counted in variety O-72 (44.44%).

Days required for shoot regeneration

There were no significant variation was recorded among different varieties of *C. olitorious* on consideration of days required for shoot regeneration under the present experiment in laboratory condition. Days required for shoot regeneration (5.40) were recorded for both the variety O-9897 and O-72 (Table 11).

4.5.2 Effect of different concentration of $FeSO_4$

Number of explants showing shoots

A statistically significant variation was recorded in considering the number of explants showing shoot induction in different concentration of $FeSO_4$. The highest number of explants showing shoots (9.83) were recorded in concentration of $FeSO_4$ 56 mg/l which was closely followed (6.67 and 6.50) by concentration of $FeSO_4$ 28 mg/l and 84 mg/l. On the other hand the lowest number of explants showing shoot (0.00) was recorded in concentration of $FeSO_4$ 0 mg/l (Plate 4).

Percent Shoot regeneration

A statistically significant variation was recorded in considering percent shoot regeneration in different concentration of $FeSO_4$. The highest percent shoot regeneration (81.94%) was recorded in concentration of $FeSO_4$ 56 mg/l which was closely followed (55.55% and 54.16%) by concentration of $FeSO_4$ 28 mg/l and 84 mg/l respectively. On the other hand, the lowest percent shoot regeneration (0.00%) was recorded in concentration of $FeSO_4$ 0 mg/l which was closely followed (33.33%) by concentration of $FeSO_4$ 112 mg/l (Table 12).

Days required for shoot regeneration

A statistically significant variation was recorded in considering the days required for shoot regeneration in different concentration of $FeSO_4$. The highest days required for shoot regeneration (7.00) were recorded in concentration of $FeSO_4$ 28 mg/l and 112 mg/l which was closely followed (6.50) by concentration of $FeSO_4$ 56 mg/l and 84 mg/l. On the other hand, the lowest days required for shoot regeneration (0.00) was recorded in concentration of $FeSO_4$ 0 mg/l (Table 12).

4.5.3 Combined effect of varieties and different concentration of $FeSO_4$

Number of explants showing shoots

The combined effect of varieties and concentration of $FeSO_4$ also showed a statistically significant difference in respect of number of explants showing shoot under the present piece of experiment. The highest number of explants showing shoot (10.00) was recorded in variety O-9897 with concentration of $FeSO_4$ 56 mg/l which statistically identical (9.67) was with variety O-72 with concentration of $FeSO_4$ 56 mg/l (Table 13). The lowest number of explants showing shoot (0.00) was counted in variety O-72 and O-9897 with concentration of $FeSO_4$ 0 mg/l.

Percent Shoot regeneration

The combined effect of variety and concentration of $FeSO_4$ also showed a statistically significant difference in respect of percent shoot regeneration under the present piece of experiment. The highest percent shoot regeneration (83.33%) was recorded in variety O-9897 with concentration of $FeSO_4$ 56 mg/l which was statistically identical (80.55%) with variety O-72 with concentration of $FeSO_4$ 56 mg/l. The lowest number of percent shoot regeneration (0.00) was counted for variety O-72 and O-9897 with concentration of $FeSO_4$ 0 mg/l (Table 13).

Days required for shoot regeneration

The combined effect of variety and concentration of $FeSO_4$ also showed a statistically significant difference in respect of days required for shoot regeneration under the present piece of experiment. The highest days required for shoot regeneration (7.00) were recorded in variety O-9897 with concentration of $FeSO_4$ 28 mg/l, 56 & 84 mg/l. The lowest days required for

shoot regeneration (0.00) was counted in variety O-72 and O-9897 with concentration of $FeSO_4$ 0 mg/l (Table 13).

Table 11. Effect of different varieties of *C. olitorious* on shoot regeneration

Varieties	No. of explants showing shoot	Percent shoot regeneration	Days required for shoot regeneration
O-72	5.33	44.44	5.40
O-9897	5.47	45.55	5.40
Level of Significance	N S	NS	NS
LSD	--	--	--

Table 12. Effect of different concentration of $FeSO_4$ of shoot regeneration on *C. olitorious*

Concentration of $FeSO_4$ (mg/l)	No. of explants showing shoot	Percent shoot regeneration	Days required for shoot regeneration
0	0.00 d	0.00 d	0.00 c
28	6.67 b	55.55 b	7.00 b
56	9.83 a	81.94 a	6.50 b
84	6.50 b	54.16 b	6.50 b
112	4.00 c	33.33 c	7.00 a
Level of Significance	**	**	**
$LSD_{0.05}$	1.301	10.84	0.012

Table 13. Combined effect of different varieties of *C. olitorious* and concentrations of $FeSO_4$ on shoot regeneration

Variety × Concentration of FeSO4 (mg/l)		No. of explants showing shoot	Percent shoot regeneration	Days required for shoot regeneration
O-72	0	0.00	0.00	0.00
	28	6.00	49.99	7.00
	56	9.67	80.55	6.00
	84	7.00	58.33	7.00
	112	4.00	33.33	7.00
O-9897	0	0.00	0.00	0.00
	28	7.33	61.11	7.00
	56	10.00	83.33	7.00
	84	6.00	49.99	6.00
	112	4.00	33.33	7.00
Level of Significance		NS	NS	NS
LSD		--	--	--

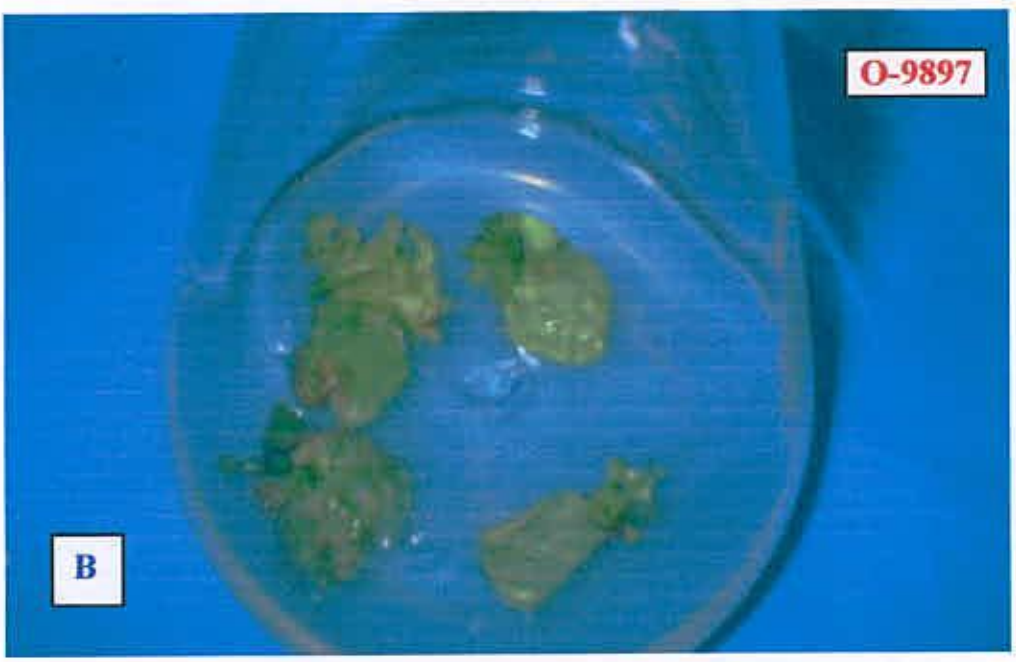


Plate 4. Comparisons of shoot regeneration of O-9897 on MS media from cotyledon of ***C. olitorius*** at different concentrations of FeSO4

Experiment 6. Effects of different level of pH on Shoot regeneration

In these experiment varieties of *C. olitorious* were cultured in pH range 4.0 to 7.5 in association with MS plant regeneration medium to know the optimum pH level for regeneration of this two varieties.

4.6.1 Effect of varieties

Percent shoots regeneration

There was a significant variation among the two varieties of *C. olitorious* on consideration of percent shoot regeneration under the present experiment in laboratory condition (Table 14). The highest percent of shoot regeneration (47.88%) were recorded for the variety O-72 and the lowest (45.95%) was recorded for variety O-9897.

No. of shoot/cotyledon

A significant variation was recorded among the two varieties of *C. olitorious* on consideration number of shoot/cotyledon under the present trial in laboratory condition. The highest no. of shoot/cotyledon (8.15) was recorded for the variety O-72. On the other hand the lowest (7.05) number of shoot/cotyledon was recorded for variety O-9897 (Table 14).

4.6.2 Effect of pH

Percent shoots regeneration

In consideration of the percent shoots regeneration there were found a statistically significant variation in different pH level (Table 15). The highest percent of shoot regeneration (65.30%) was recorded in pH level 5.5 which was closely followed by pH level 6.0. On the other hand, the lowest percent shoots regeneration (35.15%) was recorded in pH level 4.0 which was statistically identical (35.70) with pH level 7.5.

No. of shoot/cotyledon

A statistically significant variation was recorded in consideration of the number of shoot/cotyledon in different pH levels under the present experiment in laboratory condition (Table 15). The highest number of shoot/cotyledon (10.00) was recorded in pH level 6.0 which was statistically similar (9.70) by pH level 5.5. On the other hand the lowest number of shoot/cotyledon (5.40) was recorded in pH level 4.0 and 7.5 which was closely followed (6.20) by with pH level 7.0.

4.6.3 Combined effect of varieties and pH levels

Percent shoots regeneration

The combined effect of varieties and pH levels showed a statistically significant difference in respect of percent shoot regeneration under the present piece of experiment. The highest percent shoot regeneration was recorded (68.20%) were recorded in variety O-9897 with pH level 5.5. The lowest percent shoot regeneration (32.60 %) was counted in variety O-9897 with pH level 7.5 (Table 16).

No. of shoot/cotyledon

The combined effect of varieties and pH levels showed a statistically significant difference in respect of number of shoot/cotyledon under the present piece of experiment. The highest number of shoot/cotyledon was recorded (12.00) were recorded in variety O-72 with pH level 6.0. The lowest number of shoot/cotyledon (5.00) was counted in variety O-9897 with pH level 7.5 (Table 16). Comparisons of shoot regenerations of different varieties of *C. olitorius* have been presented in plate 5.

Table 14. Effect of different varieties of *C. olitorious* on percent shoots regeneration and number of shoot/cotyledon

Variety	Percent shoots regeneration	No. of shoot/cotyledon
O-9897	45.95 b	7.05 b
O-72	47.88 a	8.15 a
Level of Significance	**	**
LSD	1.403	0.241

Table 15. Effect of pH levels on percent shoots regeneration and number of shoot/cotyledon

pH levels	Percent shoots regeneration	No. of shoot/cotyledon
4.0	35.15 e	5.40
4.5	41.70 d	7.50
5.0	47.80 c	9.00
5.5	65.30 a	9.70
6.0	58.60 b	10.00
6.5	48.95 c	7.60
7.0	42.10 d	6.20
7.5	35.70 e	5.40
Level of Significance	**	**
LSD	2.806	0.482

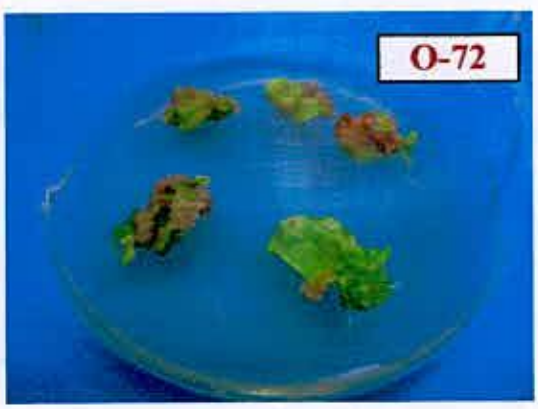
Table 16. Combined effect of varieties of *C. olitorious* and pH levels on percent shoots regeneration and number of shoot/cotyledon

Variety × pH level		Percent shoots regeneration	No. of shoot/cotyledon
O-9897	4.0	35.50 gh	5.40 gh
	4.5	40.80 fg	8.00 de
	5.0	47.40 de	8.80 d
	5.5	68.20 a	9.20 c
	6.0	56.20 c	8.00 de
	6.5	45.50 e	6.60 f
	7.0	41.40 f	5.40 fgh
	7.5	32.60 gh	5.00 gh
O-72	4.0	34.80 gh	5.40 gh
	4.5	42.60 efg	7.00 ef
	5.0	48.20 de	9.20 c
	5.5	62.40 b	10.20 b
	6.0	61.00 b	12.00 a
	6.5	52.40 d	8.60 d
	7.0	42.80 ef	7.00 ef
	7.5	38.80 g	5.80 fg
Level of Significance		**	**
LSD		3.968	0.682

A. Shoot regeneration from cotyledons of *C. olitorious* (O-9897) at pH 5.5

B. Shoot regeneration from cotyledons of *C. olitorious* (O-9897) at pH 7.5

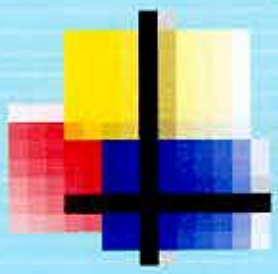
C. Shoot regeneration from cotyledons of *C. olitorious* (O-72) at pH 5.5



D. Shoot regeneration from cotyledons of *C. olitorious* (O-72) at pH 4.0

Plate 5. Comparisons of shoot regenerations of different varieties of *C. olitorius* at different P^H levels.

CHAPTER-5



SUMMARY AND CONCLUSION

Sets of experiments were conducted in the Genetic Engineering Laboratory, Cytogenetics Department, Bangladesh Jute Research Institute (BJRI), Dhaka during the period from March 2005 to May 2006 to fulfill the objectives of the study.

Detailed investigation was carried out to study the seed germination, callus induction ability and subsequent plant regeneration, root initiation of some *C. olitorious* genotypes using cotyledons (with attach petioles) as explants.

Significant variation was reported among the varieties of *C. olitorious* for germination percentage. The highest germination percentage was counted for the variety O-72 (71.68%) and the lowest was in variety O-4 (61.17%). In consideration of media for germination the highest percentage (91.58%) was counted in cotton-based liquid medium and the lowest was recorded in agar solidified medium.

Significant variations among the varieties for number of explants growing callus was recorded. The highest number of explants growing callus was recorded for the variety O-9897 (19.47) and the lowest was recorded in variety OM-1 (15.93). Considering the concentration of hormone the highest number of explants growing callus (19.96) were recorded in conc. of 3 mg/l BAP and the lowest number was recorded conc. of 4.00 mg/l BAP.

On consideration of percentage of callus induction a significant variation was recorded among the different varieties of *C. olitorious*. The highest percentage

of callus inductions was recorded for the variety O-9897 (54.07%) and the lowest was counted in variety OM-1 (44.26%). Considering the concentration of BAP the highest percent callus induction (55.43%) was recorded in conc. of 3 mg/l BAP and the lowest was recorded in conc. of 4 mg/l BAP.

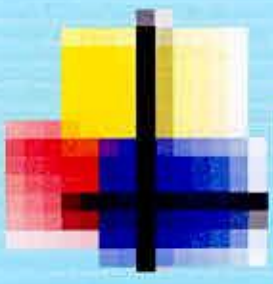
The highest average numbers of cotyledon regenerated was recorded for the variety O-72 (53.66) and the lowest number of cotyledon regenerated was counted in variety OM-1 (48.61). Considering the average number of cotyledon regenerated there was significant variation in different concentrations of BAP. The highest number of cotyledon regenerated (57.83) was recorded in conc. of 3 mg/l BAP and the lowest was recorded in conc. of. 1mg/l BAP.

Significant variation was recorded in considering the number of explants showing shoot induction in different concentration of $FeSO_4$. The highest number of explants showing shoots (9.83) recorded in conc. of 56 mg/l $FeSO_4$ and the lowest number of explants showing shoot (0.00) was recorded in conc. 0.0 mg/l $FeSO_4$.

A significant variation was recorded among the varieties of *C. olitorious* on considering percent shoot regeneration. The highest percent of shoot regeneration (47.88%) was recorded in the variety O-72 and the lowest (45.95%) was recorded for variety O-9897. Considering the percent shoots regeneration significant variations was found in different pH level. The highest percent shoot regeneration (65.30%) was recorded in pH level 5.5 and the lowest percent shoots regeneration (35.15%) was recorded in 4.0.

In conclusion, all the experiments demonstrated in this thesis a dramatic result for the seed germination from the varieties of *C. olitorius* was observed in

clinical cotton supported liquid MS medium which is very high compared to agar solidified medium. This system also ensures very healthy seedling production. The best in vitro responses for percent callus induction and percentage cotyledon producing shoots was observed in MS medium containing IAA (0.5 mg/l) and BAP (3.0 mg/l).Regeneration ability increase when MS medium incorporated with 56 mg/l $FeSO_4$ and P^H level at 5.5. This plant regeneration system could be used for genetic transformation.



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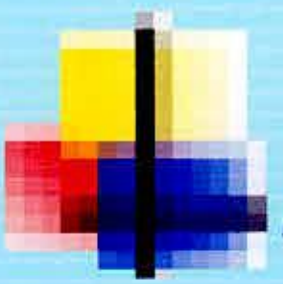

APPENDICES

Appendix I. Constituents of stock solution for MS (Murashige and Skoog, 1962) medium

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

Appendix II. Analysis of variance of the data of *C. olitorius* on number of seeds germinated and percent of seed germination

Sources of variation	Degree of freedom	Mean squares	
		No. of seed germinated	Percent seed germination
Variety (A)	3	28.81**	115.238**
Culture medium (B)	1	3566.55**	14266.201**
Interaction (A×B)	3	3.741**	14.964**
Error	16	0.280	1.118

** : Significant at 0.01 level

Appendix III. Analysis of variance of the data of *C. olitorius* on callus induction

Sources of variation	Degree of freedom	Mean squares	
		No. of explant growing callus	Percent callus induction
Variety (A)	2	40.574**	312.996**
Conc. of BAP (B)	3	24.395**	188.276**
Interaction (A×B)	6	0.366	2.837
Error	24	0.319	2.459

** : Significant at 0.01 level

Appendix IV. Analysis of variance of the data of *C. olitorius* percentage of shoot regeneration from the cotyledons (with attached petioles) of different varieties of *C. olitorius* using MS basal Medium supplemented by IAA and BAP

Sources of variation	Degree of freedom	Mean squares		
		Average number of cotyledon regenerated	% Cotyledons producing shoots	Average number of shoots produced by each cotyledon
Variety (A)	2	76.612**	191.854**	14.691**
Conc. of BAP (B)	3	238.325**	177.552**	1.337**
Interaction (A×B)	6	19.395**	11.658**	0.798**
Error	24	1.127	1.290	0.055

** : Significant at 0.01 level

Appendix V. Analysis of variance of the data of *C. olitorius* on number of shoot to which root induced, % root initiation and days to root initiation

Sources of variation	Degree of freedom	Mean squares		
		No. of shoot to which root induced	% root initiation	Days to root initiation
Variety	2	18.091**	139.583**	3.670**
Error	6	0.843	6.507	0.150

** : Significant at 0.01 level

Appendix VI. Analysis of variance of the data of *C. olitorius* on shoot regeneration

Sources of variation	Degree of freedom	Mean squares		
		No. of explant showing shoot	Percent shoot regeneration	Days required for shoot regeneration
Variety (A)	1	0.133	9.263	0.00
Conc. of hormone (B)	4	80.383**	5581.586**	55.050**
Interaction (A×B)	4	1.050	72.911	0.750
Error	20	1.167	81.018	0.0001

** : Significant at 0.01 level

Appendix VII. Analysis of variance of the data of *C. olitorius* on percent shoots regeneration and number of shoot/cotyledon

Sources of variation	Degree of freedom	Mean squares	
		Percent shoots regeneration	No. of shoot/cotyledon
Variety (A)	1	44.468**	14.52**
pH level (B)	7	680.609**	20.383**
Interaction (A×B)	7	25.589**	3.360**
Error	32	5.691	0.168

** : Significant at 0.01 level