EFFECT OF GENOTYPE, MEDIA AND EXPLANTS ON MICROPROPAGATION OF SUGARCANE

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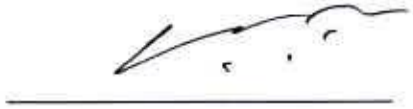

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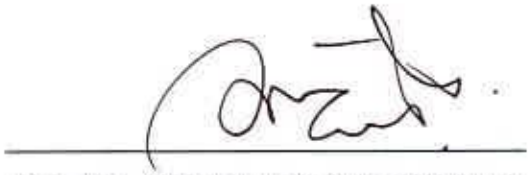

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CERTIFICATE

This is to certify that thesis entitled, "EFFECT OF GENOTYPE, MEDIA AND EXPLANTS ON MICROPROPAGATION OF SUGARCANE " 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 bonafide research work carried out by HASAN MOHAMMAD TARIQUE, Registration No. 06-02151 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.

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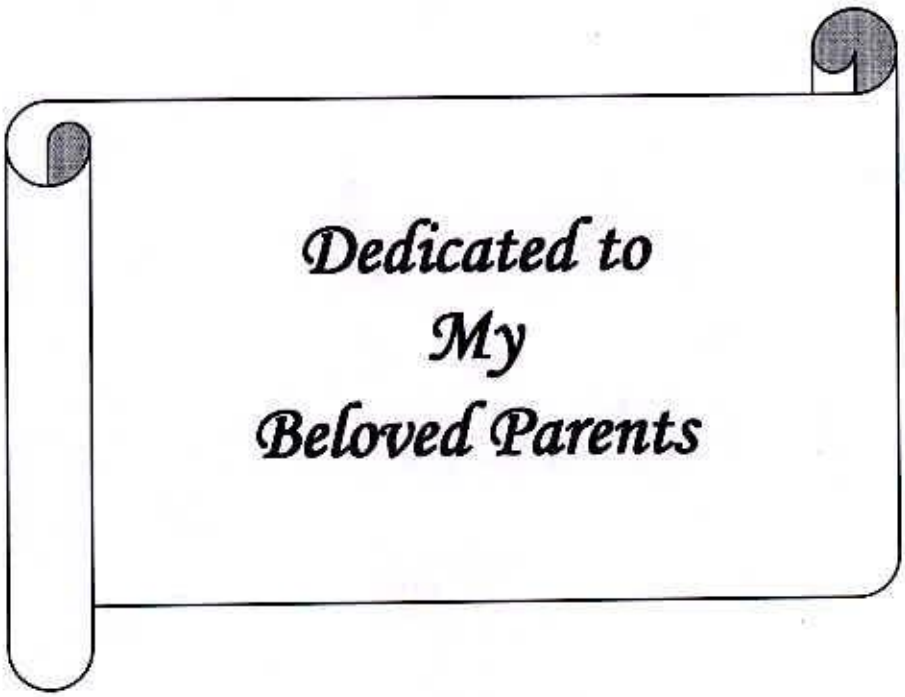
Dedicated to
My
Beloved Parents

Abbreviations of Technical Symbols and Terms

BAP	=	6-Benzyl amino purine
BSR I	=	Bangladesh Sugarcane Research Institute
CRD	=	Completely Randomized Design
DCI	=	Days of callus initiation
DSI	=	Days of shoot initiation
DRI	=	Days of root initiation
IBA	=	Indole-3-butyric acid
Kn	=	Kinetine (6 furfuryl amino purine)
MS	=	Murashige and Skoog
NAA	=	α -Naphthyl Acetic Acid
NaOH	=	Sodium Hydroxide
pH	=	Negative logarithm of hydrogen ion (H^+) concentration
2, 4-D	=	2, 4- Dichlorophenoxyacetic acid
DW	=	Distilled Water

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EFFECT OF GENOTYPE, MEDIA AND EXPLANT ON MICROPROPAGATION OF SUGARCANE

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ABSTRACT

In Bangladesh, yield of sugarcane is very low due to environmental factors including disease and insect pest infestation. Farmers need to wait for set sowing due to flood. Delayed sowing hamper the formation of sugar during maturation of sugarcane finally resulting in yield loss. Moreover, time requirement and continued infection by systemic disease are serous problems to multiply of sugarcane in the open field. The present study was conducted to select a suitable explant and protocol of micropropagation of BSRI released three sugarcane varieties. Bud, shoot tip and leaf sheath of the most important sugarcane varieties viz. Isd16, Isd36 and Isd37 were used as explants. Bud and shoot tip explants were used for direct shoot initiation and leaf sheath explants were used for callus formation followed by shoot initiation. Different concentrations of cytokinin (BAP or Kn) alone and different combinations of cytokinin (BAP) with auxin (NAA or IBA) were used with MS medium to observe their effects on shoot initiation. BAP showed better performance than Kn for shoot initiation. Effectiveness of the combination of cytokinin (BAP) with auxins (NAA or IBA) was proved to be superior to that of concentrations of cytokinin (BAP) alone. Best results were obtained from all the three varieties in MS medium supplemented with 2.0 mg/l BAP + 0.2 mg/l NAA for shoot initiation from the bud and shoot tip explants. Different concentrations of auxin (NAA and 2,4-D) were used with MS medium to observe their effects on callus formation. Best results were observed from the varieties Isd36 and Isd37 at 4.0 mg/l of 2,4-D and the variety Isd16 at 3.0 mg/l of 2,4-D for callus induction. Different combinations of BAP with NAA or IBA with MS medium were used for shoot initiation and multiplication from callus. 1.0 mg/l BAP + 0.5 mg/l NAA showed the best result for multiplication of shoots for all varieties. MS medium supplemented with different concentrations of auxin (NAA and IBA) were used alone for *in vitro* root formation from proliferated shoots. NAA showed better performance than IBA for root initiation. Best result of root formation was observed on MS medium supplemented with 5.0 mg/l of NAA. The variety Isd37 produced the highest number of roots (13.47) at 5.0 mg/l of NAA. Interactions of varieties and media exerted remarkable effects on shoot and root formation capabilities. The plantlets were successfully transferred to soil with eighty to ninety percent survivability at normal temperature with 85% humidity.

Chapter I
Introduction

CHAPTER I

INTRODUCTION

Sugarcane is the most important cash-cum-industrial crop in Bangladesh. It is a principal cash crop in North-Western and South-Western low rainfall belt of the country and the main raw material for sugar and gur industries. Sugarcane (*Saccharum officinarum* L.) is globally the main source of raw material for the production of sugar. All varieties of sugarcane are species or hybrids of the genus *Saccharum* of the family Gramineae. The genus *Saccharum* contains three cultivated species (*S. officinarum*, *S. sinensis* and *S. barberi* L) and two wild species (*S. robustum* and *S. spontanium*) (Kochhar 1998). The large barreled high sucrose containing orginal cane of *S. officinarum* is thought to have originated from the wild species *S. robustum* which is medium thick and low in sugar content (Brandes *et al.* 1939). The geographical origin of modern cultivated sugarcane (*S. officinarum*) is New Guinea and later distributed throughout the tropics and sub-tropics.

Sugarcane occupies nearly 1.5708 lac hectares (3.88 lac acre) of land in Bangladesh with a total production of 6423 lac metric tons. (Anon. 2005). The average yield of sugarcane staggers around 50 tons per hectare in the mill-zone and about 36 tons per hectare in non mill-zone areas (Anon. 1996). The average yield of cane is 45 tons per hectare against its theoretical potential of 75 tons per hectare under rainfed condition (Rahman *et al.* 1987). Both cane yield (45/ha) and recovery percentage of sugar (around 8.3 to 8.5%) are far below the average potentiality (Anon. 1993). These figures are quite low as compared to those of the advance sugarcane growing countries. Overall the country's 3 lakh metric ton of gur is produced from sugarcane, date and palm juice. But sugarcane is the greatest source of gur. It is very urgent to increase cane productivity without further area expansion to meet the future need of sugar and gur.

The sugarcane breeding progrmme has been under serious problem due to lack of suitable multiplication procedure (Lal and Singh 1994). Using conventional method usually 10-15 years of work is needed to complete a selection cycle and an improved variety can be planted commercially several years later when enough seed

canes will have been produced. Sugarcane (*Saccharum officinarum L)* like many crop plants, does not breed true type. Furthermore, it takes several years of painstaking labour to produce a pure line of sugarcane by selfing due to its high polyploidy and extreme heterozygosity (Narayanswamy 1994). In Bangladesh, sugarcane is propagated by settlings. Every year a lot of sugarcane is required as sets. Thus, farmers loss their production and sugar industries also loss sugar. Moreover, due to flood, farmers need to wait for "Jow condition" (optimum soil moisture condition for ploughing) for set sowing. Delayed sowing hampers the formation of sugar during maturation of sugarcane. If the seedlings are produced in a Biotechnology Laboratory as commercial basis, farmers can sow them immediately after the run-off of the floodwater. On the other hand, sets use for sowing can be sent to the sugar industries for sugar production. However, time requirement and continued infection by systematic disease are serious problems to multiply an elite genotype of sugarcane in the field (Lal *et.al.* 1994).

The technique of tissue culture is being routinely used for producing large number of clonal plants by *in vitro* culture of explants from wide range of species throughout the world. *In vitro* clonal propagation of plants using tissue culture techniques is popularly known as micropropagation. Now-a-days, micropropagation has become an attractive and powerful tool in the research field throughout the world, especially in the area of large scale clonal propagation, crop improvement through genetic manipulation, conservation of plant genetic resources and valuable germplasm.

To meet the future requirements of sugar it is essential to develop some improved varieties, suitable regeneration protocol for the production of virus and disease free plantlets within shorter period of time. Micropropagation is considered as a powerful tool for crop improvement within limited time period. For this purpose, development of regeneration protocols is prerequisite.

Although, sugarcane is one of the most important industrial crops, very limited effort have been made on tissue culture and *in-vitro* propagation for variety development and rapid multiplication in Bangladesh. Therefore, this investigation was made to establish the *in vitro* regeneration and rapid propagation techniques of field grown sugarcane with particular emphases given to the followings:

1. To select the best explant (s) for fast response and quick differentiation and multiplication
2. To evaluate genotypic effects on micropropagation
3. To compare the effects of media for shoot and root formation
4. To select variety specific media for plant micropropagation

Chapter II
Review of literature

CHAPTER II

REVIEV OF LITERATURE

Micropropagation through tissue culture technique has become a viable alternative of conventional clonal propagation. Research on sugarcane tissue and cell culture was initiated at the experimental station of the Hawaiian Sugar Planters Association (HSPA) in 1961 (Nickell, 1964) for physiological studies. Now-a-days micro propagation is widely used in sugarcane improvement and breeding programme.

Information on shoot proliferation, callus initiation and proliferation, shoot growth and development and root formation, relevant literature are reviewed in this chapter.

Callus was produced from *Saccharum officinarum* using mature internodal parenchyma tissue as explant (Nickell and Mertziki, 1969, Heinz *et al.* (1969). This was made possible by the pioneering work of Heinz and Mee (1969) and Barba *et. al.* (1969) who first demonstrated that plantlets could be developed for sugarcane callus culture. According to Heinz *et.al* (1969) the best medium for callus differentiation and plantlet development is that of MS medium or its modification.

Later, Heinz *et.al* (1969) and Liu *et.al.* (1976) demonstrated that sugarcane plants regenerated from callus showed wide variation in chromosomal number and in several important characters. This observation resulted in the introduction of tissue culture methods in sugarcane breeding programmes by several countries (Heinz *et al.* 1977). However, a serious problem in conventional sugarcane breeding is that it takes several years before any newly isolated variety can be released. Tissue culture is now being used for clonal propagation of a large number of economic plants including forest trees.

Callus culture of sugarcane have been successfully established using shoot apics, young leaves and young inflorescence as explant on MS medium containing 2, 4-D and coconut milk (CW) (Heinz *et al.* 1977 and Liu, 1984).

Sontichai (1988) reported that basal medium supplemented with 3 to 4 mg/l 2, 4-D and 15% coconut water was suitable for callus induction of *Saccharum spontaneum* and *Sacchrum robustum* . But cultured explant produces phenolic compound within

2-3 days and some explants became dead. Precise methods of control and maintenance of plant regeneration in sugarcane callus culture have been presented by Chen *et al.* (1998).

Vasil (1979) has shown planted regeneration to a medium low in auxin concentration or free of auxin. Somatic embryogenesis and plantlet regeneration from leaf explant have been reported by Ahloowalia (1995).

Rongrong *et al.* (1991) cultured meristem of five cultivars of sugarcane on MS medium with 3 mg/l or 1 mg/l 2,4-D inducing 10% (w/v) coconut water and was able to produce 80-100% virus free plants which could be mass propagated for further plantation in the field.

Lal *et.al.* (1991) examined the effect of 5 mg/1 2, 4-D on modified medium for callus induction and maintenance. Lower concentration of 2,4-D caused simultaneous organogenesis and embryogenesis. Dry matter production/unit weight of callus was also highest at 5 mg/1 2, 4-D.

Dhamankar (1991) reported molasses as sucrose of nutrients in plant tissue culture. He also stated that leaf explants of sugarcane CV., Co7219 were cultured in media containing 1% molasses with ammonium nitrated, coconut water, casein hydrolysate, α -naphthalene acetic acid and kinetin promoted shoot regeneration.

Rajosh *et. al.* (1994) studied *in vitro* clonal propagation of sugarcane with modified MS media supplemented with IAA, BAP and Kn having 0.5 mg/1 of each for best growth.

Lal *et.al.* (1994) said that axillary shoot proliferation from shoot tip culture is most desirable and safe micro propagation methods micropropagules minimize genetic variation. Attempts to multiply sugarcane through *in vitro* culture have been made for producing true-to-type and disease free clonal plants using meristems culture.

Lal *et.al* (1994) developed rapid multiplication of sugarcane using shoot tip culture. *In vitro* shoot multiplication and growth were optimal in modified MS medium containing 0.25 mg/1 Kn and BAP. He also observed optimal rooting and plantlet growth on semisolid half strength modified MS medium containing 0.2 mg/1 NAA. Plantlets grown on this medium for 2-3 weeks could be successfully established in

sterile sandy soil following a hardening phase of 14 days. The procedure holds promise for rapid clonal multiplication of elite sugarcane clones.

Burner and Grisham (1995) reported that propagation procedure affects the induction and stability of phenotypic variation in sugarcane. Usually high concentration (5.0mg/1) of auxins in presence of 5% sucrose helped initiation of more number (5-8) of roots in the varieties (Isd 16, Isd 28 and Amrita), indicating the necessity of readymade energy source during root initiation and their initial growth phase.

According to Heinz *et al.* (1995) the following series of steps encourages root production: 1) separate the individual plantlet and transfer into fresh agar, 2) after the shoots have developed to a few inches in height, transfer plantlets to aerated water, 3) transfer plantlets to vermiculite after the initiation of small roots. 4) transfer the rooted plants to soil under greenhouse conditions and 5) transfer to the field.

Heinz *et al.* (1995) reported that the presence of an auxin, usually 2, 4-D, but sometimes α-napthalene acetic acid (NAA) is necessary for prevention of differentiation of plantlets from callus. Use of IAA is not desirable, presumably, because of the presence IAA oxidase system in sugarcane tissues.

Synman *et al.* (1996) stated that the large proportion of white, embryogenic callus in relation to total callus volume were produced when subcultured weekly on 1.00 mg/1 2,4-D for up to six weeks. Subcultures on lmg/1 2,4-D and on variable levels of 2,4-D were less effective in callus maintenance.

Hossain *et al.* (1996) found that the highest percentage of brownish slimy callus was obtained in modified MS medium supplemented with 4.00mg/1 of 2, 4-D compared to the lower concentration of 2,4-D used. It was seen that the callus development was increased as the concentration of 2,4-D was increased in the modified MS medium and the highest concentration produced highest percent of callus.

Taylor (1997) cultured leaf explants of sugarcane cultivars on modified MS medium containing arginine, 2,4-D, coconut water. but only embryogenic and organogenic were capable of establishing embryogenic callus and high protoplast yielding suspension cultures of sugarcane.

Siddique *et al.* (1998) developed somaclones of sugarcane variety BF-162 through tissue culture using leaf as explant source. They used MS medium with different levels of 2, 4-D for studying the callogenic response. The good quality of brownish callus was obtained at 3 mg/l of 2, 4-D. For organogenesis, the callus was cultured on half MS medium supplemented with kinetin and casein hydrolysate.

Yutaka *et al.* (1998) reported that combinations of phytohormones often determine the course of morphogenesis e.g. shoot organogenesis or embryogenesis. For multiple shoot regeneration, shoot tips were remarkably influenced by types and concentration of the auxins and cytokines used. The cytokinin BAP was more effective than Kn and IBA for shoots formation. Low auxins and high cytokinin supplementation in medium favoured the induction of multiple shoot regeneration.

Mannan *et.al.* (1999) cultured shoot tips of clones Isd 16 result was observed in six weeks of culture the optimum auxillary shoots with uniform characteristics were obtained on the medium supplemented with 1.00 mg/1 Kn+1.00 mg/1 IBA+0.5 mg/1 GA_3.

Sorory *et.al.* (2000) also conducted that callus production also depends on the explant source and different genotypes require different media for callus induction.

Tripathi *et al.* (2000) conducted callusing of sugarcane variety Cose 95436 on MS medium supplemented with 5.0 mg/1 2,4-D from apical leaf roll and apical shoot meristems. *In vitro* shoot multiplication and growth were optimal at 3.0 mg/1 Kn. At this concentration of Kn, there was 8.7-fold increase of multiplication in 30 days culture, while the increase was 6.7 fold at supplemented with 1.5 mg/1.

Rahman *et al.* (2001) reported that rooting responses in stalkless settings of setting of three sugarcane varieties were studied using different concentrations (0.2, 0.5, 1.0, 2.0 and 5.0 mg/1) of NAA, IBA and IAA. Significantly higher survival of setting and rooting were found at optimum soil moisture regime when settings were soaked at 2 mg/1 NAA followed by 2mg/1 IBA. At soil moisture stress, significantly survival and rooting were observed when wettings were soaked at 5mg/1 NAA compared to other treatments.

Chattha *et al.* (2001) cultured *Saccharum* species hybrids viz. CP72-2086, CP43-33, CP77-454 and GT-11 at the Plant Genetic Resource Institute (PGRI) at National Agricultural Research Centre, Islamad. Auxillary and apical buds were cultured on

MS medium having 1.5 mg/1 concentration of BA and GA_3 Increased number of shoots and rapid shot growth were observed in variety RB 72-454.

Rahman *et al.* (2001) reported that nutrient media containing growth regulators enhanced callus induction; shoot differentiation and root formation *in vitro* of sugarcane varieties viz. Isd 20, Isd 30, Isd 2/54. All the three varieties cultured fortified with 3.0 mg/l 2,4-D showed the best performance in the callus induction on modified MS media. In shoot induction, effectiveness of cytokinin (BA 1.5 mg/l) along with 0.25 mg/1 of either NAA or IBA was proved to be superior to other concentrations. Micro shoots were rooted on modified MS media supplemented with NAA 0.5 mg/l+0.5mg/1 IBA and IBA 0.5mg/l+IAA 0.5mg/l.

Chengalrayan (2001) reported that lower concentration of BAP 0.25-1.25 mg/l showed better response on multiple shoot formation. He said that the positive effect of lower concentration of BAP on shoot proliferation of sugarcane. Higher doses of BAP suppressed the shoot proliferation.

Parwar *et.al.* (2002) obtained mass multiplication of shoots with MS medium containing IAA (0.1 mg/1) +BAP (2.0 mg/1) + Kinetin (1.0 mg/1). They also reported that phenolic secretion of sugarcane shoot culture at agar medium is always higher. However when of proliferation from the primary explant was initiated in the liquid medium and then culture was transferred to the semisolid agar medium, it resulted in better growth of shoots.

Karim *et al.* (2002) reported micro propagation oil of two sugarcane varieties viz, Isd 16 and Isd 28 through callus culture using leaf sheath as explant. The highest percentage of callus induction was observed in the medium containing 3.0 mg/l 2, 4-D with 10% coconut water. The best response in terms of multiple shoot formation was observed on NIS medium supplemented with BAP 1.0 mg/1 +IBA 0.5mg/l. NAA 3.0 mg/1 was found effective in the production of roots.

Baksha *et al.* (2002) conducted an experiment on multiple shoot production obtained from shoot tip explant of sugarcane variety Isd 28 cultured on MS medium containing BAP (0.5-2.0 mg/1), Kn (01-0.5 mg/l) and IBA (0.1-0.5 mg/1). Plant regeneration from shoot tip was highest on MS medium supplemented with BAP (2.0 ing/1) and IBA (.05 ing/1).

Karim *et al.* (2002) stated that callus was induced on modified MS medium supplemented with 2, 4-D (0.5-4.0 mg/l) as growth regulator using leaf sheath explants of sugarcane variety Isd 31. In this experiment 1.0 mg/l BAP and 0.5mg/l IBA was found superior in the optimum production of multiple shoots. NAA 5.0rng/I showed best performance in the production of roots.

Baksha *et al.* (2002) cultured anther of sugarcane clone 1 273-91 with microspores at mid uninucleate stage, respond to callusing within 60 days after planting on MS medium supplement with sucrose (3%), 2,4-D (2 mg/1) and coconut milk. They found that both albino and green plants were obtained from the androgenic calli in regeneration medium and differentiation of green plants were always more than 2,4-D induced calli in comparison to NAA induced calli. Half MS salt solution supplement with either NAA or IBA (5.00) was found suitable for rooting and further growth of the shoots. Regenerated androgenic plants showed narrow range of androgenic for different character.

Alam *et al.* (2003) reported that in-vitro morphogenesis from leaf sheath derived calli of three local varieties (Isd 16, Isd 28 and amrita) of sugarcane. The calli induced in 2,4-D (0.5 mg/L) containing MS medium showed better regenerating capacity than NAA containing one. Shoot differentiation started from the first passage of subculture in regeneration medium. reached its peak at third passage but declined beyond fifth passage of subculture. Rooting was induced in MS medium supplement with high one cone of auxin (5.00 mg/L) and sucrose (5%). Mass propagation of plantlets was attained through in-vitro tillering in 1/2 strength of liquid MS medium.

Based on their morphological appearance, two types of calli were observed: (1) type A- yellowish white, compact, dry and nodular and (II) type B- whitish globular, non compact and wet. Such type of calli has also been reported by Khan *et al.* (2003)). Best callus induction and proliferation was observed on medium containing 3 mg/l 2, 4-D.

Perveen *et al.* (2003) observed that five 2,4-D levels for calli formation and 9 combinations of growth hormones for differentiation of shoots were used keeping constant quantity of coco-milk (25 ml/l medium) in MS medium using lines /varieties viz. S 97US82, S 97US215, S 97SP23, S 97US126, CP82-1172 and CP89-

1 945 were cultured using pith parenchyma and young leaf dies as explant source. About all the genotypes respond well to caullogenesis on three levels (1, 3, 5 mg/1) of 2, 4-D and genotypes CP82-1172 gave maximum callus at all 2, 4-D levels. Maximum regeneration was observed at 1 and 3 mg/1 2, 4-D levels by about all the genotypes using leaf callus for regeneration. Maximum number of shoots was developed at the combination 480 mg/I (Casein Hydrolysate) +1.00 mg/1 (Kinetin) +25 rnl/l of coco-milk.

Ali *et.al.* (2003) cultured three variety of sugarcane and stated that NSG-6 and NSG-311) had given maximum shoots (3.5 and 3.8 per that so culture, respectively) in the media combination where Kn 0.1 +BAP 1.0 mg/1was used however vigorous growth was observed in the combination where Kn 0. +BAP 1.5 mg/1 was applied. For the rooting NSG-6 and NSG-555 respond better (64 and 51% respectively) in the hormonal combination of IBA +NAA. It was also observed that NSG-555 gave more root length in the treatment where 4 mg/I of IBA +NAA were used.

Karim *el al.* (2003) suggested that callus formation from leaf sheath explain was observed within 3 weeks of incubation on modified MS medium supplemented with 2, 4-D (1.0-4.0 mg/1) and coconut mill at room temperature. Effective calli were formed at 3.0 mg/1 2, 4-D and 10% coconut milk oil medium. Best shoot regeneration was observed at BAP (1.0 nig/1) +NAA (0.5 mg/1) on MS medium. Regenerated shoots were rooted on both MS and half strength of MS medium supplemented with NAA.

Khan (2003) worked out seven sugarcane clone and showed that highest callus formation and plantlet regeneration were recorded in clone NIA-98 while the lowest in CP67-412 followed by SPSG-26. The maximum chlorophyll mutation frequency was noted in clone AEC82-1028 and minimum in AEC81-0819. Best root induction was observed medium containing MS+1 mg/1 IBA + 6% sucrose.

Rahman *et al.* (2003) studied for callus induction and plant regeneration were carried out oil four sugarcane genotypes viz. LHO83-153, LCP81-10, CPF-236 and CPF-237 using MS medium with 1,3,5,7 and 9 mg/I 2,4-D was used for callus induction and leaf and pith as a explant source. Leaf, as explant source with average callus score 2.77 was better than pith (1. 00). Genotype LCP 81-10 was the best callus producer and CPF-237 proved to be the poor callus producer 1.61. Among the

five 2, 4-D levels the 1.00 and 3.00 mg/l gave better result and LCP81-10 produced maximum shoots per test tube (9.35) where as LHO proved to the poor regeneration (1.55 shoots per test tube)

Ahmed *et al.* (2004) cultured two different types of explants such as shoot tips and nodal buds (eye) of in vitro grown sugarcane (Saccharum officinarum) variety CO-527. Multiple shoot regeneration was found on MS medium supplemented with 0.5 mg/l BAP and 0.75 mg/l NAA. Roots were induced when the isolated individual shoot was cultured on MS medium containing 7.5 mg/l NAA. More than 80% plantlets thus obtain were successfully established on to the soil under natural condition.

Sabaz *et al.* (2008) carried out an experiment for rapid micropropagation of three sugarcane varieties i.e., HSF-240, CP-77-400 and CPF-237. Shoot tip was used as explant source. Shoot initiation from explant of all three varieties was achieved at 1 mg/l Kin and 0.1 mg/l GA3. For rapid multiplication the regenerated shoots were transferred on liquid Murashige and Skoog medium containing 2% sucrose, supplemented with BAP in combinations with GA3. Optimum multiplication was observed at 1 mg/l BAP in combination with 0.1 mg/l GA3 for variety HSF-240. Best response of multiplication for variety CP-77-400 was observed at 0.5 mg/l BAP with 0.1 mg/l GA3. Variety CPF-237 was multiplied at 1.0 mg/l BAP with 0.5 mg/l GA3. Rooting response was observed on half strength liquid MS medium with 6% sucrose containing different concentrations of IBA and NAA. The sugarcane plantlets were acclimatized in greenhouse.

Chapter III
Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 Location

The experiment was carried out at the Laboratory of the Breeding Division, Bangladesh Sugarcane Research Institute (BSRI) Ishurdi, Pabna during the period from August 2007 to May 2008. The materials and methods used on this investigation are described below:

3.2 Plant materials

Buds, shoot tips and leaf sheaths of sugarcane (*Saccharum officinarum*) were used as experimental materials. The explants viz. bud, shoot tip and leaf sheath were collected from 3 - 4 months aged sugarcane plants from the BSRI experimental field. These explants were cultured aseptically for *in vitro* propagation on MS (Murashige and Skoog, 1962) medium with different concentrations and combinations of growth regulators. The plants materials from three sugarcane varieties viz. Isd16, Isd36 and Isd37 were used in different experiments.

3.3 Chemicals and sources

All chemical compounds used as macro-nutrients and micro-nutrients in the present study were reagent grade (GPR) product of either Riedel-de-Haen, Germany; BDH, England/India or E. Mark, Germany/India. The vitamins and growth regulators were mostly products of Sigma Chemical Company, USA. A small portion of them was produced from the BDH, England.

3.4 Surface sterilants and surfactants

In the present investigation mercuric chloride ($HgCl_2$) was used as surface sterilizing agent while Savlon 3%(w/v) was used as detergent cum surfactant.

3.5 Preparation of stock solutions of culture media

The stock solutions of different ingredients were prepared as the first step of the media preparation practice. The various constituents of the medium were prepared into stock solutions for ready use during the preparation of the media for different experiments. As different constituents were required in different concentrations

Table 1. Composition and concentrations used for the preparation of MS media

Constituents	Concentration mg/l
A. Macro nutrients	
KNO_3	1900.00
NH4NO3	1650.00
KH2PO4	170.00
CaCl2/2H2O	440.00
MgSO4/7H2O	370.00
B. Micro-nutrients	
MnSO4.4 H_2O	22.30
H3BO3	6.20
ZnSO4.7 H_2O	8.60
KI	0.83
Na2MoO4.4 H_2O	0.25
CuSO4.5 H_2O	0.025
$CoC_{l3}.6H_2O$	0.025
C. Iron Source	
FeSO4.7 H_2O	27.80
Na2-EDTA	37.30
D. Organic nutrients (100x)	
Glycine	2.00
Nicotinic acid	0.50
Pyrodoxine HCl	0.50
Thimine HCl	0.10
Myo-inositol	100.00

Separate stock solutions of the macronutrients, micro-nutrients, iron, vitamins, growth regulators etc. were prepared and used.

Chemical composition of MS (Murashige and Skoog 1962) medium was prepared following the composition mentioned in the Table-1 and media preparation was carried out following the different steps mentioned below.

3.5.1 Stock solution A (Macro nutrients)

Stock solution of macronutrients was prepared 10 times of desired concentration with distilled water (DW). Required amount of salts were taken in a conical flask, following the serial number mentioned in Table 1 and were dissolved in distilled water (DW) using a magnetic stirrer. Finally, required amount of DW was added up to the mark. This solution was filtered and poured into a clean brown glass bottle, labeled with marker and stored in a refrigerator at 4 ± 1^{0}C for use.

3.5.2 Stock solution B (Micro nutrients)

The stock solution of micro nutrients was made to 100 times higher strength than required concentration of the medium in one liter of DW as described earlier for the stock solution of macronutrients. This stock solution was also filtered, labeled and stored in refrigerator at 4 ± 1^{0}C.

3.5.3 Stock solution C (Iron source)

The stock solution of iron source was made 100 times higher than the final strength of the medium in one liter of DW. Here, two constituents, FeSO4 and Na_2EDTA, were dissolved in 750 ml of DW in a conical flask by heating in a water bath or on a heater cum magnetic stirrer until the salts dissolved completely and final volume was made up to one liter by further addition of DW. This stock solution was also filtered and stored in refrigerator at 4 ± 1^{0}C.

3.5.4 Stock solution D (Vitamins and amino acids)

The following vitamins and amino acids were used in the preparation of MS medium:

1. Pyridoxine HCl (Vitamin B_6),
2. Thiamin HCl (Vitamin B_1),
3. Nicotinic acid (Vitamin B_3).

4. Glycine and
5. Myoinositol (Inositol).

Each of the above vitamins and amino acids were made into stock solution separately. The stock solutions of vitamins were prepared 100 times the concentrations of their final strength and stored at 4 ± 1^0C.

3.5.5 Stock solution E (Plant Growth Regulators/PGR)

Separate stock solutions of PGR'S were prepared by dissolving the desired quantity of ingredients to the appropriate solvent and made the final volume with distilled water. The following growth regulators were used in the present investigation and were dissolved in solvents specified after their names.

Plant growth regulators (solute)	Solvent
2, 4-D	50% ethyl alcohol
BAP	0.1 N NaOH
Kinetin	0.1 N HCl
NAA	0.1 N NaOH
IBA	0.1 N NaOH

To prepare the stock solution, 10 mg of growth regulator was taken in a 100 ml clean beaker and then dissolved in 1 ml of respective solvent mentioned above. Then the volume was made up to 100 ml by adding DW to get 100 mg/l solution. The prepared hormone stock solution was then stored at 4 ± 1^0C for subsequent use.

3.7 Miscellaneous

Others substances like sucrose and agar were added directly to the media during preparation as carbon source, gelling agent and absorbent respectively.

3.8 Steps followed for the preparation of culture media

To prepare one liter of MS medium, the following steps were followed:

⇒ One hundred ml of macronutrients, 10 ml of micronutrients, 100 ml of irons and 10 ml of vitamins were taken from each of these stock solutions into 2 liter Erlenmeyer flasks on a heater cum magnetic stirrer.

⇒ Five hundred ml distilled water was added in the flask to dissolve all the ingredients.

⇒ One hundred mg of Myo-inisitol was added directly to the solution and dissolved well.

⇒ Thirty gm of sucrose was added to this solution and gently agitated with help of a magnetic stirrer to dissolve completely.

⇒ Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well. Since, 100 ml of hormonal stock solution contained 10 mg of hormonal salts, the addition of 10 ml of any hormonal stock solution to prepare 1 liter of medium resulted in 1mg/1 concentration of that hormonal supplements.

The whole mixture was then made up to 1000 ml with further addition of distilled water.

⇒ p^H of the medium was adjusted to 5.7 with a digital pH meter with the help of 0.lN NaOH or 0.lN HCl, whichever was necessary.

⇒ After adjusting the pH meter, 7gm agar was added to solidify the medium. The mixture was then gently heated with continuous stirring till complete dissolution of agar.

⇒ 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 non absorbent cotton and marked with different codes with the help of a glass marker to indicate specific hormonal combinations.

3.9. Sterilization

In *in vitro* techniques, an aseptic condition is a prerequisite. So, all instruments, glassware and culture media were sterilized following appropriate procedures.

3.9.1. Sterilization of culture medium

The culture test tubes, flasks containing the medium were autoclaved with 1.16 kg/cm^2 of pressure at 121^0C for 20 minutes. After autoclaving the culture test tube and flasks containing the culture medium were allowed to cool.

3.9.2. Sterilization of glassware's and other instruments

Breakers, conical flasks, test tube, pipettes, petri dishes, metal instruments viz. forceps, scalpels, needles and spatulas were sterilized by wrapping with aluminum foils in an autoclave at a temperature of 121^0C for 20 minutes at 1.16 kg/cm^2 pressure.

3.9.3. Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all floors and walls with a detergent and germicide like Savlon or Dettol. This was followed by carefully wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals. Generally laminar airflow cabinet was sterilized by switching on the cabinet with UV light and wiping the working surface with 70% ethyl alcohol.

3.10. Precautions to ensure aseptic condition

To maintain and ensure aseptic condition precautions were taken in every step of works. All inoculation and aseptic manipulation were carried out by using a laminar airflow cabinet. It was usually switched on half an hour before use. The cabinet was wiped with 70% ethyl alcohol (C_2H_5OH) to reduce the chances of contamination. The inoculation instruments like scalpels, forceps etc. were sterilized by autoclaving and again sterilized by using hot bed sterilizer and cooling inside the inoculation chamber before use. Other required materials like distilled water, hard board etc. were sterilized by autoclave.

Hands were properly washed with soap before starting work in laminar air flow cabinet. During the operation hands were rubbed with 70% ethyl alcohol frequently with cotton and wiped cabinet base for maintaining clean condition. To obtained possible contamination free condition care was taken in clean bench during explants preparation. Aseptic condition was maintained during each and every operation to avoid contamination.

3.11. Culture techniques

The under mentioned techniques and steps were followed in the investigation for regeneration of the complete plantlets of *Saccharum officinarum*.

3.11.1. Source, collection, surface sterilization and preparation of explants

Healthy, disease free three types of explants viz. bud, shoot tip and leaf sheath were taken from the field grown canes of the varieties viz. Isd16, Isd36 and Isd37. The canes after collection from the field, and snapping of the leaves were brought to the laboratory for leaf sheath, shoot tip and bud explants. First of all the young canes were washed thoroughly under running tap water and then outer green leaf sheaths were removed. From these materials creamy white leaf sheath was taken for surface sterilization. From the top of the cane, leaf sheaths with shoot tip were cut into segments (approximately 2.0 cm) and prepared as explant.

Prepared explants were taken in a beaker and treated with 1% Savlon for 5-6 minutes with constant shaking and were thoroughly washed with distilled water for 2-3 minutes. The explants were transferred in autoclaved plastic pot and treated with 0.1% mercuric chloride ($HgCl_2$) followed by 4-5 times rinsed with sterile distilled water to remove traces of $HgCl_2$ from the explants.

3.11.2. Inoculation of explants (Bud and Shoot tip) for direct regeneration

The buds and shoot tip explants were dissected with the help of sterilized forceps and scalpels. The plugs of the culture vessels were removed inside laminar airflow cabinet and the explants were inoculated on the culture medium. One explant was cultured into each culture tube for assessing initial response of the explant to auxillary shoot proliferation. After inoculation the culture vessels were plugged near the sprite lamp and labeled by glass marker with inoculation date. Proliferating cultures of auxillary shoots were established on suitable medium from the Bud and Shoot tip and inoculation on different aspects of shoot proliferation was noted down accordingly.

3.11.3. Subculture for multiplying shoots cultures

When the explants grew into small, leafy structure ,they were rescued aseptically from the culture tube and transferred into fresh medium containing the same hormonal combination or best one among them for further proliferation and development. Subculture carried out regularly at an interval of 4-5 weeks.

3.11.4 Inoculation of the explants (Leaf sheath) for indirect regeneration

Leaf sheath segments were prepared on the laminar air flow cabinet aseptically from the sterilized explants using a fine sterile forceps and scalpel. The excised explants were then inoculated into each culture test tubes containing MS media with various concentration and combination of hormonal supplement for *in vitro* micropropagation.

3.11.5. Incubation

The culture vessels with inoculated explants were incubated both in dark and light in a temperature controlled growth room (25±1^{O}C) under 16 hours photoperiod illuminated fluorescent tube of 2000-3000 lux. Day to day observations was carried out to note the response.

3.11.6. Callus culture

Explants were cultured for homogeneous callus on MS media supplemented with different concentrations of NAA and 2,4-D for three to four weeks. Three varieties and 3 replications (each comprised 10 test tubes) were used for each treatment and each test tube contained one sample.

3.117. Shoot culture

When good callus was formed, then the calli were aseptically transferred into fresh medium containing the different hormonal treatments for proliferation and development of shoot.

3.11.8. Subculture

Successful shoot formation became evident when small green fresh leaves began to emerge. It is the first sign of regeneration. These tiny leaves when developed in their actual shape were transferred into fresh medium containing the same hormonal combination or best one among them for further proliferation and development. Subculture carried out regularly at an interval of 4-5 weeks.

3.11.9. Plantlet culture

Regenerated mini plantlets were cultured on media supplemented with NAA (0.5, 1.0, 3.0 and 5.0 mg/l) or IBA(0.5, 1.0, 3.0 and 5.0 mg/l) individually for root formation. The subcultured shoot continued to proliferate and in some of the

cultures they differentiated into shoots. When these shoots grew about 3-5 cm in length, with 2-3 well developed leaves they were rescued aseptically from the test tube and were separated from each other and again cultured on freshly prepared medium containing different concentrations of NAA.

3.12. Data Collection

1) Days to shoot initiation from the bud explants.
2) Days to shoot initiation from the shoot tip explants.
3) Days to callus initiation from the leaf sheath explants.
4) % of explants induced callus from the leaf sheath explants.
5) Days to shoot initiation from the callus.
6) Number of shoot per culture.
7) Number of useable shoot per culture.
8) Shoot length.
9) Days to root initiation.
10) Number of roots per shoot.
11) Root length.

3.13. Statistical analysis of data

The data for the characters under the present study were statistically analyzed following Completely Randomized Design (CRD). Results were assessed by standard analysis of variance using computer program MSTAT-C (MSTAT Development Team, 1988).

Chapter IV
Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

The present study was conducted to develop a suitable reproducible protocol for rapid clonal propagation of *Saccharum officinarum* through tissue culture technique. Micropropagation technique is one of the most successful examples of commercial application of tissue culture that provides viable alternative method for mass production of healthy plants with uniform characteristics (Lim and Kong, 1985). It is also possible to propagate a huge number of plantlets from a single explant within a shortest span of time with the help of this technique (*Bajaj et al.*, 1992). Technique for direct or indirect plantlet regeneration *in vitro* following the steps of shoot proliferation, callus formation, adventitious shoots differentiation and rooting of the micro-shoots have been established through this investigation on the three commercial varieties of sugarcane viz. Isd 16, Isd 36 and Isd 37. The results obtained from different experiments of the present investigation are described and tabulated under the following headings.

4.1. Direct Regeneration

Direct regeneration of complete plantlets was achieved through bud (eyes) culture. The direct regeneration is said to be the useful means of regenerating plantlets from young and mature plants with a lower risk of genetic instability than by other routes (Rao and Lee 1986).

4.1.1. Effects on shoot proliferation from the bud explant

The bud explant of three varieties of sugarcane were inoculated to five different types of concentrations and four different types combination of media for differentiation and proliferation of shoots. It was observed that the number of shoots production per culture and shoot length differed significantly among varieties and shooting media. Data are presented on the Tables 2-7 and plate no. 1. Days to shoot initiation were not differed significantly.

4.1.2. Effects on days to shoot initiation (DSI)

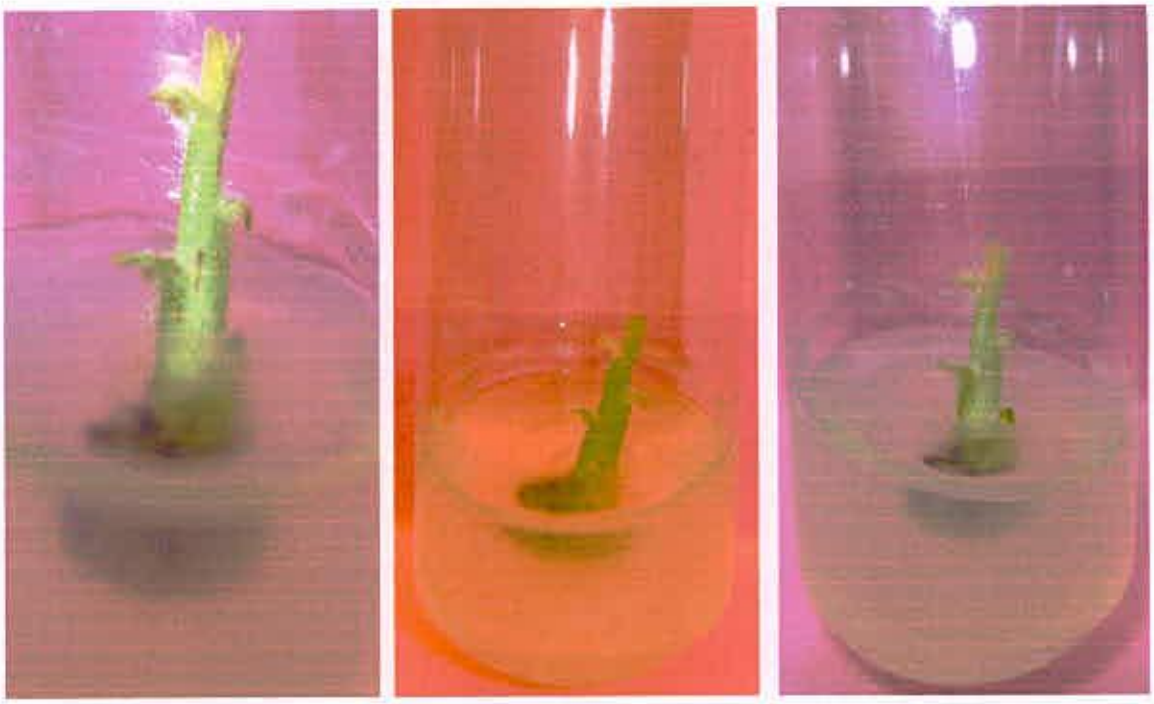
Among the factors, varietal effect was not significant for days of shoot initiation whereas shoot media was significant. The effect of varieties on days of shoot initiation was found insignificant. The shoot initiation days required for variety Isd37 was the lowest (33.33) and the highest days (33.57) required to shoot initiation was in Isd36 and the variety Isd16 was showed statistically identical result (Table-2).

The effects of shooting media were significant for days to shoot initiation. The lowest days (31.11) required for shoot initiation was in the MS medium supplement with $BA_{2.0}$. This result was significantly lower over that of other treatments. The days required for $BA_{1.0}$, $BA_{1.5}$, $BA_{2.5}$, $BA_{3.0}$, $Kn_{1.0}$, $Kn_{1.5}$, $Kn_{2.0}$, $Kn_{2.5}$ and $Kn_{3.0}$ were 33.89, 33.67, 33.33, 33.67, 33.89, 33.78, 32.33, 34.22 and 34.33 (Table-3) respectively. The highest days (34.33) required for shoot initiation was in the MS medium supplement with $Kn_{3.0}$. These results indicated that $BA_{2.0}$ was the best performance for days to shoot initiation.

Combined effects of variety and shoot media on production of shoot for days to shoot initiation were presented on the Table 4. The variety Isd36 and Isd37 were produced shoot within the lowest days (31.00) on shooting media $BA_{2.0}$ containing MS medium. The highest days (34.67) required for the shoot initiation was in the variety Isd16 and MS medium supplemented with $Kn_{2.5}$. In this experiment, Isd36 or Isd37 and $BA_{2.0}$ was the best combination for shoot initiation.

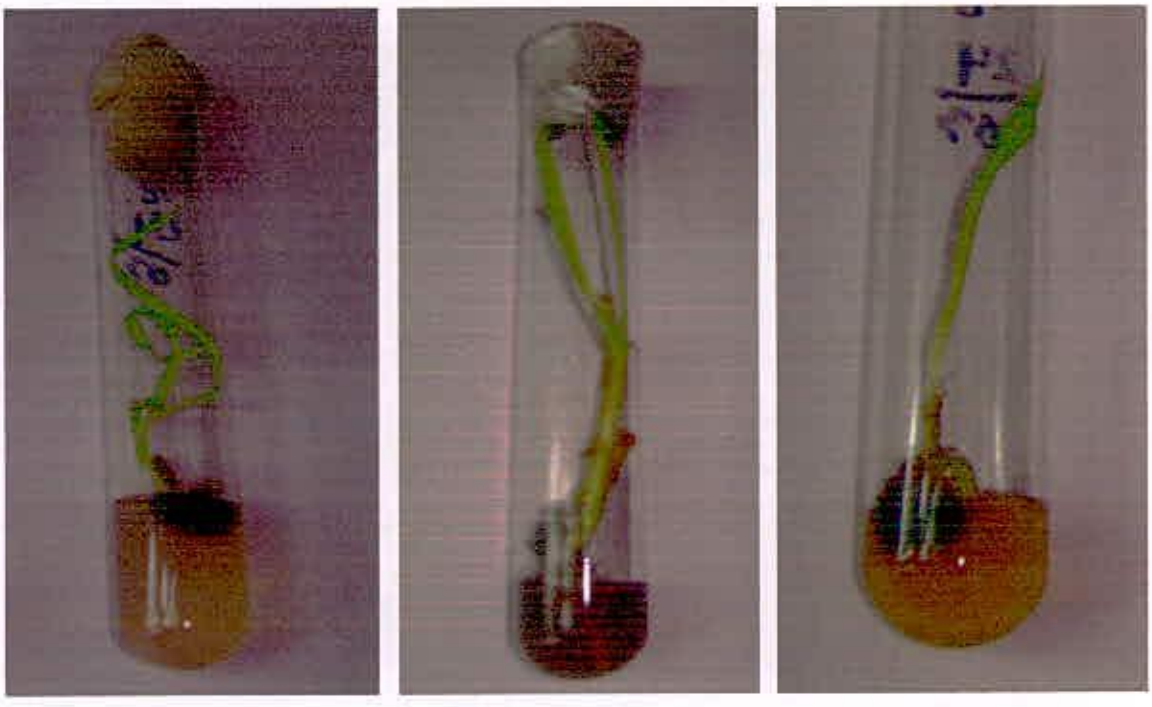
The results of the other four different types combinations and concentrations of shooting media are presented in Table 5-7. In this experiment, the effect of varieties was found not significant. The shoot initiation days required for variety Isd37 was the lowest days (34.75) and the highest days (35.17) required to shoot initiation was Isd16 and the variety Isd36 was showed statistically identical result (Table-5).

The effects of combinations of shooting media were significant for days to shoot initiation. The lowest days (33.44) required for shoot initiation was in the MS medium supplement with $BA_{2.0}NA_{0.2}$(Table-6). This result was significantly higher over that of other treatments. The days required for $BA_{2.0}NA_{0.1}$, $BA_{2.0}NA_{0.5}$, $BA_{2.0}NA_{1.0}$, $BA_{2.0}IB_{0.1}$, $BA_{2.0}IB_{0.2}$, $BA_{2.0}IB_{0.5}$ and $BA_{2.0}IB_{1.0}$ were 35.22, 35.67,



Isd16 Isd36 Isd37

Plate 1.1: Shoot proliferation from the bud explants of sugarcane



Isd16 Isd36 Isd37

Plate 1.2: Growth of initiated shoot from bud explant of sugarcane

35.56, 34.78, 33.56, 35.78 and 35.78 respectively. These results indicated that $BA_{2.0}NA_{0.2}$ and $BA_{2.0}IB_{0.2}$ were the best performance for days to shoot initiation.

Interaction effects of variety and combination of shoot media on production of shoot for days to shoot initiation were presented on the Table 7. The variety Isd36 produced shoot within the lowest days (33.00) on shooting media $BA_{2.0}IB_{0.2}$ containing MS medium. The highest days (36.33) required for the shoot initiation was in the variety Isd16 and MS medium supplemented with $BA_{2.0}NA_{1.0}$. In this experiment, Isd36 or Isd37 and $BA_{2.0}IB_{0.2}$ was the best combination for shoot initiation.

4.1.3. Effects on number of shoot per culture

Highly significant result was obtained for shoot per culture incase of the main effect of variety production of shoot from the Table 2; the highest number of shoot (0.25) was observed in the variety Isd37 followed by the variety Isd16 with statistically identical result. Lowest number of shoots (0.20) was found in the variety Isd36. So, the variety Isd 37 showed effective performance for shoot production. This value was highly significant over that of other varieties.

Highly significant result was observed for shoot per culture incase of shooting media on shoot production. The MS media supplemented with $BA_{2.0}$ which produced the highest number of shoots (0.47) per culture followed by $Kn_{2.0}$ produced shoots 0.31 (Table-3). The number of shoots per culture 0.16, 0.29, 0.32, 0.13, 0.18, 0.31 and 0.22 were obtained in the media supplemented with $BA_{1.0}$, $BA_{1.5}$, $BA_{2.5}$, $BA_{3.0}$, $Kn_{1.5}$, $Kn_{2.0}$ and $Kn_{2.5}$ respectively. Lowest performance (0.10) was observed with $Kn_{1.0}$ or $Kn_{3.0}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}$ was the best performing treatment.

No significant result was found in the combined effect of variety and shooting media for shoot per culture. Isd37 produced highest number (0.50) of shoots per culture in the MS medium supplemented with $BA_{2.0}$ (Table-4). The lowest number of shoot (0.10) was observed in the variety Isd36 with shooting medium $BA_{3.0}$ or $Kn_{1.0}$ or Kn 3.0. From this investigation Isd37 and MS medium supplemented with $BA_{2.0}$ showed the best performance among the variety and shooting media respectively.

Table 2: Responses of varieties for direct regeneration from the bud explants under different cytokinin (BA and Kn) concentrations

Variety	Days to shoot initiation	No. of shoot per culture	Shoot length (cm)
Isd 16	33.37	0.24	2.86
Isd 36	33.57	0.20	2.80
Isd 37	33.33	0.25	3.12
LSD (0.05)	0.623	0.023	0.071

Table 3 : Effects of cytokinin (BA and Kn) at different concentrations in MS medium for direct regeneration from the bud explant.

Concentration of growth regulator (mg/l)	Days to shoot initiation	No. of total shoot per culture	Shoot length (cm)
BA 1.0	33.89	0.16	2.77
BA 1.5	33.67	0.29	3.09
BA 2.0	**31.11**	**0.47**	**3.43**
BA 2.5	33.33	0.32	3.17
BA 3.0	33.67	0.13	2.91
Kn 1.0	33.89	0.10	2.52
Kn 1.5	33.78	0.18	2.73
Kn 2.0	**32.33**	**0.31**	**3.21**
Kn 2.5	34.22	0.22	2.86
Kn 3.0	34.33	0.10	2.60
LSD (0.05)	1.138	0.042	0.130

Table 4 : Combined effects of varieties and cytokinin (BA and Kn) concentrations for direct regeneration from the bud explant

Variety X Cytokinin (mg/l)	Days to shoot initiation	No. of shoots per culture	Shoot length (cm)
Isd 16 X BA 1.0	33.67	0.17	2.70
X BA 1.5	33.00	0.30	3.10
X BA 2.0	**31.33**	**0.47**	**3.37**
X BA 2.5	33.33	0.33	3.13
X BA 3.0	33.33	0.13	2.80
X Kn 1.0	33.67	0.10	2.47
X Kn 1.5	33.67	0.20	2.67
X Kn 2.0	32.67	0.33	3.07
X Kn 2.5	34.67	0.23	2.80
X Kn 3.0	34.33	0.10	2.53
Isd 36 X BA 1.0	34.00	0.13	2.63
X BA 1.5	34.33	0.23	3.07
X BA 2.0	31.00	0.43	3.40
X BA 2.5	33.00	0.30	3.07
X BA 3.0	34.67	0.10	2.83
X Kn 1.0	34.00	0.10	2.37
X Kn 1.5	34.00	0.17	2.53
X Kn 2.0	32.33	0.27	3.03
X Kn 2.5	34.00	0.17	2.70
X Kn 3.0	34.33	0.10	2.37
Isd 37 X BA 1.0	34.00	0.17	2.97
X BA 1.5	33.67	0.33	3.10
X BA 2.0	**31.00**	**0.50**	**3.53**
X BA 2.5	33.67	0.33	3.30
X BA 3.0	33.00	0.17	3.10
X Kn 1.0	34.00	0.10	2.73
X Kn 1.5	33.67	0.17	3.00
X Kn 2.0	32.00	0.33	3.53
X Kn 2.5	34.00	0.27	3.07
X Kn 3.0	34.33	0.10	2.90
LSD (0.05)	1.971	0.073	0.225

Table 5 : Responses of varieties for direct regeneration from the bud explants under various combinations of cytokinin (BA) with auxin (NAA or IBA)

Variety	Days to shoot initiation	No. of shoots per culture	Shoot length (cm)
Isd 16	35.17	0.30	2.86
Isd 36	35.00	0.28	2.84
Isd 37	34.75	0.33	2.90
LSD (0.05)	0.497	0.025	0.058

Table 6 : Effects of cytokinin (BA) with auxin (NAA or IBA) for direct regeneration from the bud explant on MS media

Cytokinin (mg/l) + Auxin (mg/l)	Days to shoot initiation	No. of shoot per culture	Shoot length (cm)
$B_{2.0}NA_{0.1}$	35.22	0.31	3.00
$B_{2.0}NA_{0.2}$	**33.44**	**0.56**	**3.40**
$B_{2.0}NA_{0.5}$	35.67	0.34	3.07
$B_{2.0}NA_{1.0}$	35.56	0.21	2.66
$B_{2.0}IB_{0.1}$	34.78	0.24	2.53
$B_{2.0}IB_{0.2}$	33.56	0.39	3.06
$B_{2.0}IB_{0.5}$	35.78	0.26	2.73
$B_{2.0}IB_{1.0}$	35.78	0.12	2.49
LSD (0.05)	0.813	0.042	0.094

Table 7 : Combined effects of varieties and cytokinin (BA) with auxins (NAA or IBA) for direct regeneration from the bud explant.

Variety X Cytokinin + Auxin (mg/l)	Days to shoot initiation	No. of shoots per culture	Shoot length (cm)
Isd 16 X $B_{2.0}NA_{0.1}$	35.00	0.33	3.00
$B_{2.0}NA_{0.2}$	33.33	0.53	3.43
$B_{2.0}NA_{0.5}$	36.00	0.37	3.10
$B_{2.0}NA_{1.0}$	36.33	0.23	2.73
$B_{2.0}IB_{0.1}$	35.00	0.23	2.53
$B_{2.0}IB_{0.2}$	34.00	0.37	3.03
$B_{2.0}IB_{0.5}$	35.67	0.27	2.73
$B_{2.0}IB_{1.0}$	36.00	0.10	2.30
Isd 36 X $B_{2.0}NA_{0.1}$	36.00	0.27	2.97
$B_{2.0}NA_{0.2}$	33.67	0.53	3.30
$B_{2.0}NA_{0.5}$	35.67	0.33	2.97
$B_{2.0}NA_{1.0}$	35.33	0.20	2.60
$B_{2.0}IB_{0.1}$	34.67	0.23	2.70
$B_{2.0}IB_{0.2}$	33.00	0.37	3.00
$B_{2.0}IB_{0.5}$	35.67	0.20	2.73
$B_{2.0}IB_{1.0}$	36.00	0.10	2.47
Isd 37 X $B_{2.0}NA_{0.1}$	34.67	0.33	3.03
$B_{2.0}NA_{0.2}$	**33.33**	**0.60**	**3.47**
$B_{2.0}NA_{0.5}$	35.33	0.33	3.13
$B_{2.0}NA_{1.0}$	35.00	0.20	2.63
$B_{2.0}IB_{0.1}$	34.67	0.27	2.37
$B_{2.0}IB_{0.2}$	33.67	0.43	3.13
$B_{2.0}IB_{0.5}$	36.00	0.30	2.73
$B_{2.0}IB_{1.0}$	35.33	0.17	2.70
LSD (0.05)	1.408	0.073	0.164

The results of the other four different type combination and concentration of shooting media are presented on Table 5-7. In this experiment, the effect of varieties was found highly significant. The highest number of shoot (0.33) was observed in the variety Isd37 followed by the variety Isd16 was 0.30. Lowest number of shoots (0.28) was found in the variety Isd36. So, the variety Isd37 showed effective performance for shoot production. This value was highly significant over that of other varieties.

Highly significant result was observed for shoot per culture incase of combination of shooting media on shoot production. The MS media supplemented with $BA_{2.0}NA_{0.2}$ which produced the highest number of shoots (0.56) per culture. The number of shoots per culture 0.31, 0.34, 0.21, 0.24, 0.39 and 0.26 were obtained in the media supplemented with $BA_{2.0}NA_{0.1}$, $BA_{2.0}NA_{0.5}$, $BA_{2.0}NA_{1.0}$, $BA_{2.0}IB_{0.1}$, $BA_{2.0}IB_{0.2}$ and $BA_{2.0}IB_{0.5}$ respectively. Lowest performance (0.12) was observed with $BA_{2.0}IB_{1.0}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}NA_{0.2}$ was the best performing treatment.

No significant result was found in the interaction effects of variety and combination of shooting media for shoot per culture. Isd37 produced highest number (0.60) of shoots per culture in the MS medium supplemented with $B_{2.0}$ NA $_{0.2}$(Table-7). The lowest number of shoot (0.10) was observed in the variety Isd16 and Isd36 with combination of shooting medium $B_{2.0}$ $IB_{1.0}$. From this investigation Isd37 and MS medium supplemented with $BA_{2.0}NA_{0.2}$ showed the best performance among the variety and combination of shooting media respectively.

4.1.4. Effects on shoot length

Shoot length depended on varieties, shooting media. Among varieties, shooting media and combination of shooting media and their interactions, main effects of varieties, shooting media, interaction effects of varieties and shooting media had remarkable effects on shoot length.

Highly significant result was obtained for shoot length in case of the main effect of variety on shoot production. The variety Isd37 showed highest shoot length (3.12 cm) was significantly higher over other varieties. The lowest shoot length (2.80 cm) was found in the varieties Isd16 and intermediate was Isd36. All varieties gave the

better shoot length. These results indicate that the variety Isd37 was the best for increasing shoot length (Table-2).

Shooting media also affected the shoot length. The highest shoot length (3.43 cm) was found in the medium containing $BA_{2.0}$. and other treatments were also better performance for shoot length (Table-3). The lowest shoot length (2.52 cm) was obtained from the $Kn_{1.0}$ in MS medium. Here, MS medium supplemented with $BA_{2.0}$ was the best performing treatment.

Interaction effects of the variety and shoot media for shoot length were not significant. The variety Isd37 showed highest shoot length (3.53) in the $BA_{2.0}$ or $Kn_{2.0}$ in MS medium. The lowest shoot length (2.37 cm) was found in the variety Isd36 and MS medium containing $Kn_{1.0}$ or $Kn_{3.0}$ alone (Table-4). From this investigation Isd37 and MS medium supplemented with $BA_{2.0}$ showed the best performance among the variety and shooting media respectively.

The results of the other four different types of combinations and concentrations of shooting media are presented on Table 5-7. In this experiment, the effect of varieties was not found significant. The highest shoot length (2.90 cm) was observed in the variety Isd37 followed by the variety Isd16 was 2.86 cm (Table-5). Lowest shoot length (2.84 m) was found in the variety Isd36. So, the variety Isd37 showed effective performance for shoot production. This value was not significant over that of other varieties.

Highly significant result was observed for shoot length incase of combination of shooting media on shoot production. The MS media supplemented with $BA_{2.0}NA_{0.2}$ which produced the highest shoot length (3.40 cm) (Table-6). The shoot length 3.0cm, 3.07cm, 2.66cm, 2.53cm, 3.06cm and 2.73cm were obtained in the media supplemented with $BA_{2.0}NA_{0.1}$, $BA_{2.0}NA_{0.5}$, $BA_{2.0}NA_{1.0}$, $BA_{2.0}IB_{0.1}$, $BA_{2.0}IB_{0.2}$ and $BA_{2.0}IB_{0.5}$ respectively. Lowest performance (2.49cm) was observed with $BA_{2.0}IB_{1.0}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}NA_{0.2}$ was the best performing treatment.

Highly significant result was found in the Interaction effects of variety and combination of shooting media for shoot length. Isd37 produced highest shoot length (3.47cm) in the MS medium supplemented with $B_{2.0}$ NA $_{0.2}$ (Table-7). The lowest shoot length (2.30cm) was observed in the variety Isd16 with combination of

shooting medium $B_{2.0}$ $IB_{1.0}$. From this investigation Isd37 and MS medium supplemented with $BA_{2.0}NA_{0.2}$ showed the best performance among the variety and combination of shooting media respectively.

During the present investigation it was observed that the bud explants were cultured on MS medium with different concentrations and combination of growth regulators. The explants cultured with only cytokinin failed to give satisfactory proliferation and elongation of axillary shoots. On the other hand, the explants cultured with both cytokinin and auxin combinations showed elongation of shoots. The results of the present investigation agreed with the findings of Hendra *et al.* (1983), Lal and Singh (1990) and Dhamankar (1992) who found that the explants with meristematic zone/bud produce plantlets in vitro on Murashige and Skoog's (1962) and modified MS medium containing cytokinins and auxins in different concentrations and combinations.

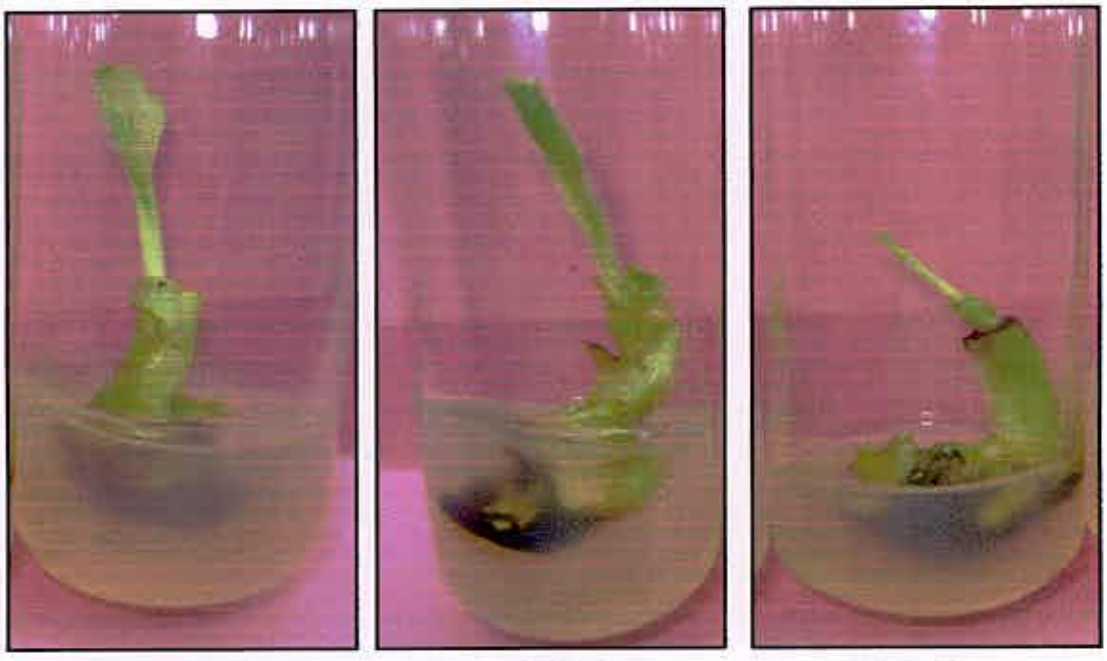
4.2. Effects on shoot proliferation from the shoot tip explant

The shoot tip explant of three varieties of sugarcane were inoculated to five different concentrations and four different combinations of media for differentiation and proliferation of shoots. It was observed that the number of shoot production per culture and shoot length were differed significantly among varities and shooting media. Days to shoot initiation did not differ significantly. Data are presented on Tables 8-13 and Plate no. 2 – 2.2.

4.2.1. Effects on days to shoot initiation (DSI)

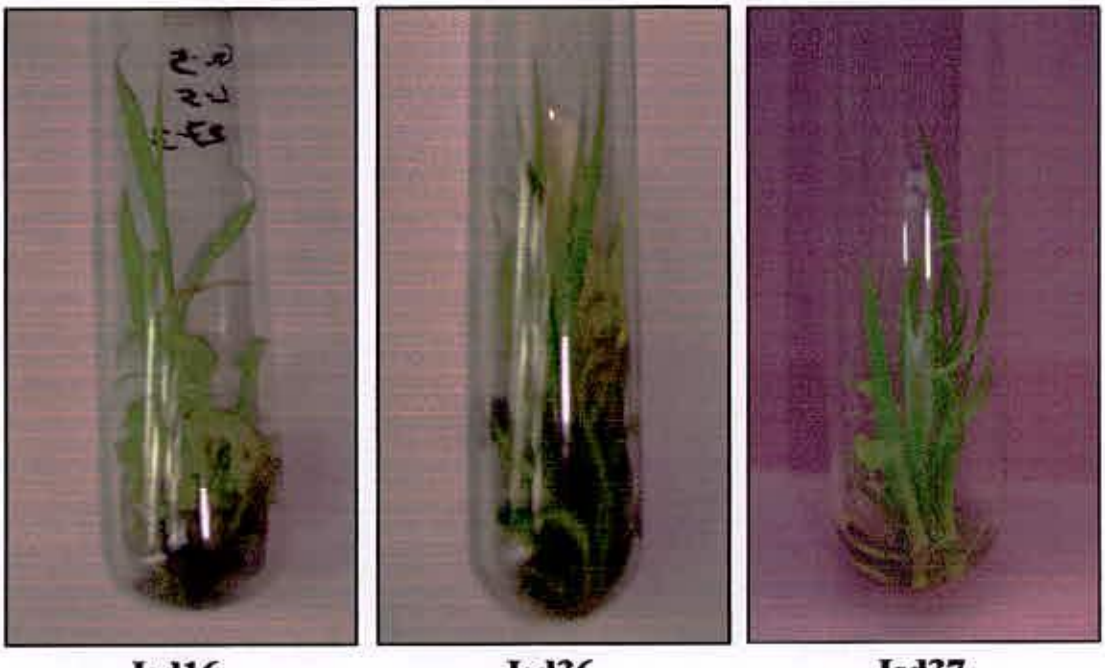
For days to shoot initiation among the factors varietal effect was not significant and shoot media was found significant. The effects of varieties on days to shoot initiation were not found significant. The shoot initiation days required for variety Isd37 was the lowest (16.00) and the highest days (16.33) required to shoot initiation was Isd36 and the variety Isd16 showed (16.30) statistically identical result (Table-8).

The effects of shooting media were significant for days to shoot initiation. The lowest days (15.11) required for shoot initiation was in the MS medium supplement with $Kn_{2.0}$(Table-9). This result was significantly lower over that of other treatments. The days required for $BA_{1.0}$, $BA_{1.5}$, $BA_{2.0}$, $BA_{2.5}$, $BA_{3.0}$, $Kn_{1.0}$, $Kn_{1.5}$ and $Kn_{2.5}$ were 16.44, 15.44, 15.46, 16.00, 17.00, 16.56, 16.33 and 16.22 respectively.



Isd16 Isd36 Isd37

Plate 2.1: Initiation of shoot from the shoot tip explants of sugarcane



Isd16 Isd36 Isd37

Plate 2.2: Growth of initiated shoot from the shoot tip explant of sugarcane

The highest days (17.44) required for shoot initiation was in the MS medium supplement with $Kn_{3.0}$. These results indicated that $Kn_{2.0}$ was the best performance for days of shoot initiation.

Combined effects of variety and shoot media on production of shoot for days to shoot initiation presented on the Table 10. The variety Isd37 were produced shoot within the lowest days (14.33) on shooting media $BA_{1.5}$ containing MS medium. The highest days (17.67) required for the shoot initiation was in the variety Isd16 & Isd36 and MS medium supplemented with $Kn_{1.5}$ or $Kn_{3.0}$ respectively. In this experiment, Isd37 and $BA_{1.5}$ was the best combination for shoot initiation.

The results of the other four different type of combination and concentration of shooting media are presented on Table 11-13. In this experiment, the effect of varieties was found not significant. The shoot initiation days required for variety Isd37 was the lowest days (16.21) and the highest days (16.54) required to shoot initiation was Isd36 and the variety Isd16 was showed statistically identical result (Table-11).

The effects of combinations of shooting media were significant for days to shoot initiation. The lowest days (15.00) required for shoot initiation was in the MS medium supplement with $BA_{2.0}NA_{0.2}$ or $BA_{2.0}IB_{0.2}$.(Table-12). This result was significantly higher over that of other treatments. The days required for $BA_{2.0}NA_{0.1}$, $BA_{2.0}NA_{0.5}$, $BA_{2.0}IB_{0.1}$, and $BA_{2.0}IB_{0.5}$ and were 16.22, 16.67, 16.56 and 16.33 respectively. The highest days (17.56) required for shoot initiation was in the MS medium supplement with $BA_{2.0}NA_{1.0}$ or $BA_{2.0}IB_{1.0}$. These results indicated that $BA_{2.0}NA_{0.2}$ and $BA_{2.0}IB_{0.2}$ were the best performance for days of shoot initiation.

Interaction effects of variety and combination of shoot media on production of shoot for days to shoot initiation were presented on the Table-13. The variety Isd37 was produced shoot within the lowest days (14.67) on shooting media $BA_{2.0}IB_{0.2}$ containing MS medium. The highest days (17.67) required for the shoot initiation was in the variety Isd36 and MS medium supplemented with $BA_{2.0}NA_{1.0}$ or $BA_{2.0}IB_{1.0}$. In this experiment, Isd37 and $BA_{2.0}IB_{0.2}$ was the best combination for shoot initiation.

4.2.2. Effects on number of shoot per culture

Table 8: Responses of varieties for direct regeneration from the shoot tip explants under different cytokinin (BA and Kn) concentrations

Variety	Days to shoot initiation	No. of shoot per culture	Shoot length (cm)
Isd 16	16.30	2.34	3.34
Isd 36	16.33	2.37	3.20
Isd 37	16.00	2.53	3.30
LSD (0.05)	0.388	0.051	0.049

Table 9 : Effects of cytokinin (BA and Kn) at different concentrations in MS medium on direct regeneration from the shoot tip explant.

Concentration of growth regulator (mg/l)	Days to shoot initiation	No. of shoot per culture	Shoot length (cm)
BA 1.0	16.44	2.31	3.22
BA 1.5	15.44	3.06	3.51
BA 2.0	15.46	3.53	4.03
BA 2.5	16.00	2.67	3.57
BA 3.0	17.00	2.28	2.71
Kn 1.0	16.56	1.81	2.64
Kn 1.5	16.33	2.23	3.40
Kn 2.0	15.11	2.72	3.87
Kn 2.5	16.22	1.99	3.13
Kn 3.0	17.44	1.53	2.72
LSD (0.05)	0.710	0.094	0.089

Table 10. Combined effects of varieties and cytokinin (BA and Kn) on direct regeneration from the shoot tip explant.

Variety X Cytokinin (mg/l)	Days to shoot initiation	No. of shoots per culture	Shoot length (cm)
Isd 16 X BA 1.0	16.33	2.13	3.13
BA 1.5	16.33	2.97	3.80
BA 2.0	14.67	3.60	4.13
BA 2.5	15.67	2.60	3.57
BA 3.0	16.67	2.33	2.93
Kn 1.0	16.67	1.70	2.57
Kn 1.5	17.67	2.03	3.50
Kn 2.0	15.33	2.73	3.90
Kn 2.5	16.33	1.80	3.20
Kn 3.0	17.33	1.53	2.70
Isd 36 X BA 1.0	16.67	2.30	3.03
BA 1.5	15.67	3.07	3.70
BA 2.0	16.67	3.37	4.00
BA 2.5	15.67	2.70	3.53
BA 3.0	17.00	2.40	2.70
Kn 1.0	16.67	1.67	2,63
Kn 1.5	16.00	2.17	3.20
Kn 2.0	15.33	2.50	3.73
Kn 2.5	16.00	2.03	2.90
Kn 3.0	17.67	1.50	2.53
Isd 37 X BA 1.0	16.33	2.50	3.50
BA 1.5	14.33	3.13	3.03
BA 2.0	15.33	3.63	3.97
BA 2.5	16.67	2.70	3.60
BA 3.0	17.33	2.10	2.50
Kn 1.0	16.33	2.07	2.73
Kn 1.5	15.33	2.50	3.50
Kn 2.0	14.67	2.93	3.97
Kn 2.5	16.33	2.13	3.30
Kn 3.0	17.33	1.57	2.93
LSD (0.05)	1.230	0.163	0.109

Table 11: Responses of varieties for direct regeneration from the shoot tip explants under various combinations of cytokinin (BA) with auxin (NAA or IBA)

Variety	Days to shoot initiation	No. of shoots per culture	Shoot length (cm)
Isd 16	16.30	3.19	3.24
Isd 36	16.54	2.96	3.09
Isd 37	16.21	3.32	3.34
LSD (0.05)	0.313	0.073	0.095

Table 12. Effects of cytokinin (BA) with auxin (NAA or IBA) on direct regeneration from the shoot tip explant on MS media

Cytokinin (mg/l) + Auxin (mg/l)	Days to shoot initiation	No. of shoot per culture	Shoot length (cm)
$B_{2.0}NA_{0.1}$	16.22	3.86	3.69
$B_{2.0}NA_{0.2}$	15.00	4.40	4.14
$B_{2.0}NA_{0.5}$	16.67	4.01	3.86
$B_{2.0}NA_{1.0}$	17.56	3.38	3.17
$B_{2.0}IB_{0.1}$	16.56	1.96	2.63
$B_{2.0}IB_{0.2}$	15.00	3.21	3.11
$B_{2.0}IB_{0.5}$	16.33	2.37	2.78
$B_{2.0}IB_{1.0}$	17.56	2.07	2.39
LSD (0.05)	0.512	0.119	0.155

Table 13. Combined effects of varieties and cytokinin (BA) with auxins (NAA or IBA) on direct regeneration from the shoot tip explant

Variety X Cytokinin + Auxin (mg/l)	Days to shoot initiation	No. of shoots per culture	Shoot length (cm)
Isd 16 X $B_{2.0}NA_{0.1}$	16.33	3.93	3.63
$B_{2.0}NA_{0.2}$	15.00	4.50	4.13
$B_{2.0}NA_{0.5}$	16.33	4.03	3.83
$B_{2.0}NA_{1.0}$	17.33	3.37	3.07
$B_{2.0}IB_{0.1}$	17.00	2.03	2.70
$B_{2.0}IB_{0.2}$	15.00	3.23	3.10
$B_{2.0}IB_{0.5}$	16.33	2.37	2.87
$B_{2.0}IB_{1.0}$	17.33	2.07	2.57
Isd 36 X $B_{2.0}NA_{0.1}$	16.33	3.60	3.60
$B_{2.0}NA_{0.2}$	1500	4.13	4.03
$B_{2.0}NA_{0.5}$	17.00	3.77	3.70
$B_{2.0}NA_{1.0}$	17.67	3.13	3.03
$B_{2.0}IB_{0.1}$	16.67	1.80	2.47
$B_{2.0}IB_{0.2}$	15.33	3.03	3.10
$B_{2.0}IB_{0.5}$	16.67	2.23	2.60
$B_{2.0}IB_{1.0}$	17.67	1.97	2.17
Isd 37 X $B_{2.0}NA_{0.1}$	16.00	4.03	3.83
$B_{2.0}NA_{0.2}$	15.00	4.57	4.27
$B_{2.0}NA_{0.5}$	16.67	4.23	4.03
$B_{2.0}NA_{1.0}$	17.67	3.63	3.40
$B_{2.0}IB_{0.1}$	16.00	2.03	2.73
$B_{2.0}IB_{0.2}$	14.67	3.37	3.13
$B_{2.0}IB_{0.5}$	16.00	2.50	2.87
$B_{2.0}IB_{1.0}$	17.67	2.17	2.43
LSD (0.05)	0.887	0.207	0.269

Highly significant result was obtained for shoot per culture incase of the main effect of variety on production of shoot from the Table 8; the highest number of shoot (2.53) was observed in the variety Isd37. Lowest number of shoots (2.34) was found in the variety Isd16 followed by the variety Isd36 with statistically identical result. So, the variety Isd37 showed effective performance for shoot production. This value was highly significant over that of other varieties.

Highly significant result was observed for shoot per culture incase of shooting media on shoot production. The MS media supplemented with $BA_{2.0}$ which produced the highest number of shoots (3.53) per culture followed by $BA_{1.5}$ produced shoots 3.06 (Table-9). The number of shoots per culture 2.31, 2.67, 2.28, 1.81, 2.23, 2.72 and 1.99 were obtained in the media supplemented with $BA_{1.0}$, $BA_{2.5}$, $BA_{3.0}$, $Kn_{1.5}$, $Kn_{2.0}$ and $Kn_{2.5}$ respectively. Lowest performance (1.53) was observed with $Kn_{3.0}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}$ was the best performing treatment.

No significant result was found in the combined effect of variety and shooting media for shoot per culture. Isd37 produced highest number (3.63) of shoots per culture in the MS medium supplemented with $BA_{2.0}$ (Tabe-10). The lowest number of shoot (1.5) was observed in the variety Isd36 with shooting medium $Kn_{3.0}$. From this investigation Isd37 and MS medium supplemented with $BA_{2.0}$ showed the best performance among the variety and shooting media respectively.

The results of the other four different types of combination and concentration of shooting media are presented in Table 11-13. In this experiment, the effect of varieties was found highly significant. The highest number of shoot (3.32) was observed in the variety Isd37 followed by the variety Isd16 (3.19) (Table-11). Lowest number of shoots (2.96) was found in the variety Isd36. So, the variety Isd37 showed effective performance for shoot production. This value was highly significant over that of other varieties.

Highly significant result was observed for shoot per culture incase of combination of shooting media on shoot production. The MS media supplemented with $BA_{2.0}NA_{0.2}$ which produced the highest number of shoots (4.40) per culture (Table-12). The number of shoots per culture 3.86, 4.01, 3.38, 3.21, 2.37 and 2.07 were obtained in the media supplemented with $BA_{2.0}NA_{0.1}$, $BA_{2.0}NA_{0.5}$, $BA_{2.0}NA_{1.0}$,

$BA_{2.0}IB_{0.2}$, $BA_{2.0}IB_{0.5}$ and $BA_{2.0}IB_{1.0}$ respectively. Lowest performance (1.96) was observed with $BA_{2.0}IB_{0.1}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}NA_{0.2}$ was the best performing treatment.

No significant result was found in the interaction effects of variety and combination of shooting media for shoot per culture. Isd37 produced highest number (4.57) of shoots per culture in the MS medium supplemented with $B_{2.0}NA_{0.2}$ (Table-13). The lowest number of shoot (1.80) was observed in the variety Isd36 with combination of shooting medium $BA_{2.0}$ $IB_{0.1}$. From this investigation Isd37 and MS medium supplemented with $BA_{2.0}NA_{0.2}$ showed the best performance among the variety and combination of shooting media respectively.

4.2.3. Effects on shoot length

Shoot length is depends on varieties and shooting media. Among varieties, shooting media and combination of shooting media and their interactions, main effects of varieties, shooting media, interaction effects of varieties and shooting media have remarkable effects on shoot length.

Highly significant result was obtained for shoot length in case of the main effect of variety. The variety Isd16 showed highest shoot length (3.34 cm) was significantly higher over other varieties, followed by the variety Isd37 with statistically identical result (Table-8). The shoot length (3.20 cm) was found in the variety Isd36. All varieties gave the better shoot length. These results indicate that the variety Isd16 was the best for increasing shoot length.

In case of the main effect of shooting media was observed highly significant for the shoot length. The MS media supplemented with $BA_{2.0}$ which produced the highest shoot length (4.03cm) (Table-9). The shoot lengths 3.22cm, 3.51cm, 3.57cm, 2.71cm, 3.40cm, 3.87cm, 3.13cm, 2.72cm were obtained in the media supplemented with $BA_{1.0}$, $BA_{1.5}$, $BA_{2.5}$, $BA_{3.0}$, $Kn_{1.5}$, $Kn_{2.0}$, $Kn_{2.5}$ and $Kn_{3.0}$respectively. Lowest performance (2.64) was observed with $Kn_{1.0}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}$ was the best performing treatment.

Interaction effects of the variety and shoot media for shoot length were highly significant. The variety Isd16 showed highest shoot length (4.13cm) on shooting media $BA_{2.0}$ containing MS medium (Table-10). The lowest shoot length (2.50cm) was found in the variety Isd37 and MS medium containing $BA_{3.0}$ alone. From this

investigation Isd16 and MS medium supplemented with $BA_{2.0}$ showed the best performance among the variety and shooting media respectively.

The results of the other four different type combination and concentration of shooting media are presented on Table 11-13. In this experiment, the effect of varieties was found highly significant. The highest shoot length (3.34cm) was observed in the variety Isd37 followed by the variety Isd16 was 3.24cm (Table-11). Lowest shoot length (3.09cm) was found in the variety Isd36. So, the variety Isd37 showed effective performance for shoot length. This value was highly significant over that of other varieties.

Highly significant result was observed for shoot length incase of combination of shooting media. The MS media supplemented with $BA_{2.0}NA_{0.2}$ which produced the highest shoot length (4.14cm) (Table-12). The shoot lengths 3.69cm, 3.86cm, 3.17cm, 2.63cm, 3.11cm and 2.78cm were obtained in the media supplemented with $BA_{2.0}NA_{0.1}$, $BA_{2.0}NA_{0.5}$, $BA_{2.0}NA_{1.0}$, $BA_{2.0}IB_{0.1}$, $BA_{2.0}IB_{0.2}$ and $BA_{2.0}IB_{0.5}$ respectively. Lowest performance (2.39cm) was observed with $BA_{2.0}IB_{1.0}$ containing MS medium. Here MS medium supplemented with $BA_{2.0}NA_{0.2}$ was the best performing treatment.

Not significant result was found in the Interaction effects of variety and combination of shooting media for shoot length. Isd37 produced highest shoot length (4.27cm) in the MS medium supplemented with $B_{2.0}NA_{0.2}$ (Tale-13). The lowest shoot length (2.17cm) was observed in the variety Isd36 with combination of shooting medium $B_{2.0}$ $IB_{1.0}$. From this investigation Isd37 and MS medium supplemented with $BA_{2.0}NA_{0.2}$ showed the best performance among the variety and combination of shooting media respectively.

From the results of the present investigation, it was observed that shoot proliferation from the shoot tip were highly affected by the types and concentrations of the auxins and cytokinin. The cytokinin BA was more effective than Kn. For multiple shoot regeneration, combination types of growth regulators (BA + NAA or IBA) was highly effective than single type (BA or IBA). Similar observation obtained from many scientist who found that the shoot tip explant produce plantlet in vitro on MS and modified MS medium containing different cytokinins and auxins in different concentrations and combinations (Grishan *et al.* 1989 and Jimenes *et al.* 1990).

However this finding agreed with Yutaka *et al.* (1998) who reported that for multiple shoot regeneration, shoot tips were remarkably influenced by types and concentration of the auxins and cytokinines used. Low auxin and high cytokinin supplementation in medium favoured the induction of multiple shoot regeneration.

4.3. INDIRECT REGENERATION

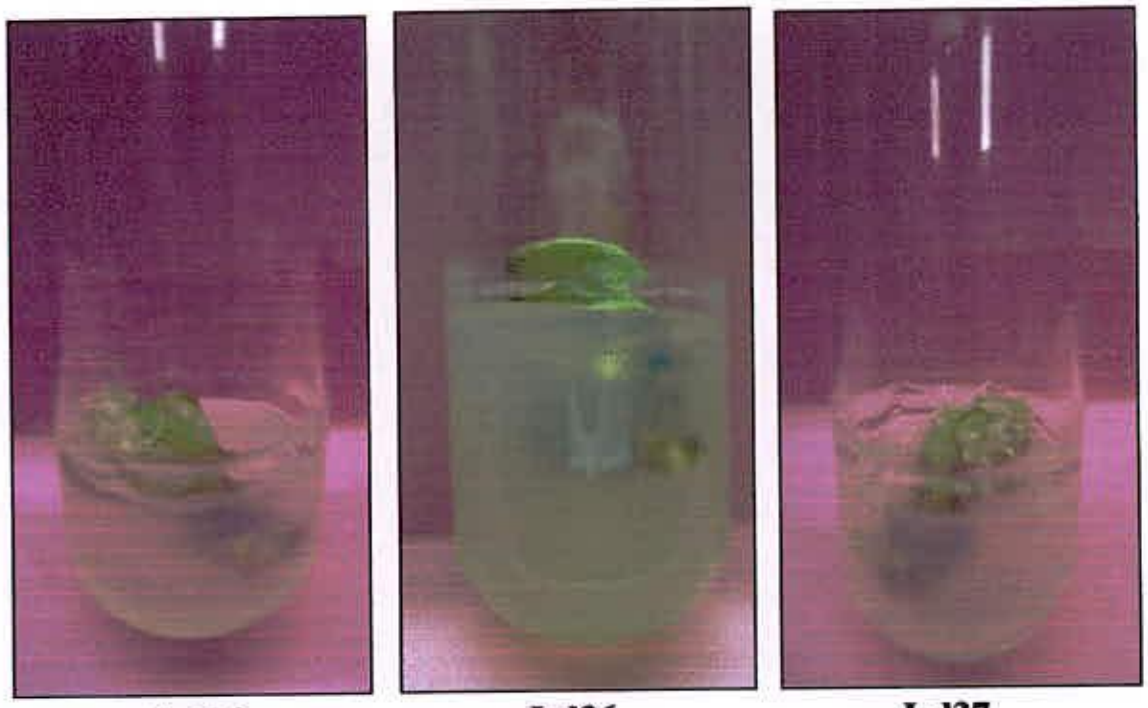
High frequency of plant regeneration from callus of leaf sheath explant offers a feasible propagation method in sugarcane which can be utilized for the year round, rapid, pathogen free and quality plantlet production. It is also be useful for preservation of valuable germplasm and for the future crop improvement programme.

In the present investigation, the techniques for plantlet regeneration *in vitro* through callus induction, shoot regeneration, multiplication and rooting of the shoots have been established very carefully using leaf sheath explant of three varieties of sugarcane viz. Isd16, Isd36 and Isd37. The results obtained from different experiments of the present study are presented and discussed in this chapter with Tables and Plates under the following headings.

4.3.1. Callus induction

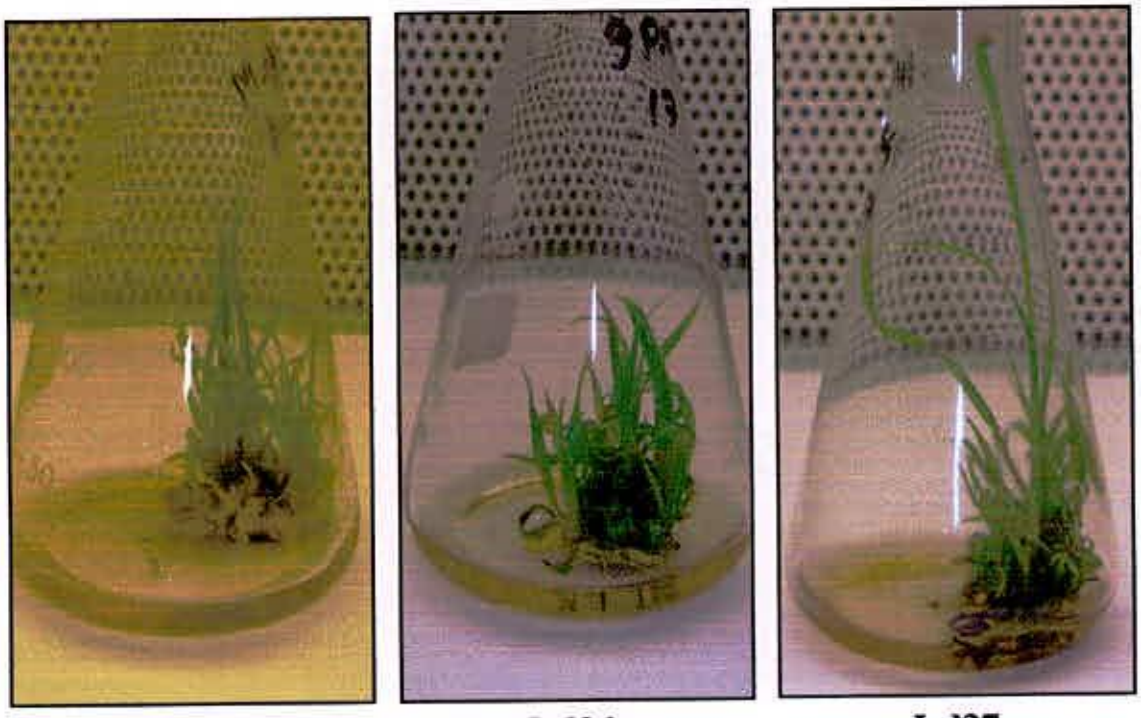
The surface sterilized leaf sheath explants of sugarcane varieties viz. Isd16, Isd36 and Isd37 were inoculated on MS medium supplemented with different concentrations of 2, 4-D and NAA separately for callus induction. Callus initiation was observed after 10-15 days of culture and calli grew vigorously until the nutrient of the medium became exhausted.

After 15 days, it was observed that inclusion of 2, 4-D in MS medium was found essential for callus initiation and the effects of NAA on callus initiation was very low. The highest amount of callus was formed in the variety Isd37 followed by Isd36. The lowest amount of callus was recorded in the variety Isd16. Among the concentrations of 2, 4-D, 4.0 mg/l produced the highest amount of callus. Response of varieties with different concentrations of 2,4-D on callus induction have been shown on Tables 14, 15,16.



Isd16 Isd36 Isd37

Plate 3.1: Formation of callus from the leaf sheath explants of sugarcane.



Isd16 Isd36 Isd37

Plate 3.2: Growth of initiated shoots from callus tissue of sugarcane

4.3.2. Effects on callus formation

Effects of varieties, auxin (NAA and 2,4-D) concentrations and their interactions on days to callus initiation and percentage of explants showed callus induction are presented on Tables 14-16 and Plate no.3.1 to 3.2.

4.3.3. Effects on days of callus initiation (DCI)

The main effect of variety under different auxin (NAA and 2,4-D) concentrations was significant of days of callus imitation. Varietal differences are existed for days to callus initiation. Initiation of callus is started with differentiation cells. Hormone present in the varieties plays the key role to initiated callus on explants. Different varieties required variables days of callus initiation perhaps due to their internal hormonal effects. The lowest days (18.53) required of callus initiation was found in the variety Isd37 and the highest days (18.97) required in the variety Isd36 (Table-14).

The days of callus initiation was also affected by different concentration of auxins (NAA and 2,4-D) supplemented into medium. Among different concentrations of NAA and 2,4-D, the results of days of callus initiation with NAA were very lower than 2,4-D concentrations. So, we here discuss about effects of 2,4-D concentration.

The main effect of 2,4-D on callus initiation in three sugarcane varieties was found significant. The lowest days (25.56) required for callus initiation in 2.0 mg/l 2, 4-D (Table-15). On the contrary, the highest days (27.44) required for callus initiation in 1.0 mg/l 2,4-D. MS medium containing 2,4-D 3.0 mg/l and 5.0 mg/l gave the similar result. So the best result was observed in the 4.0 mg/l 2,4-D. This value was significantly superior over other treatments. The maximum amount of callus initiation was observed at 4.0 mg/l and the minimum at 1.0 mg/l of 2, 4-D in all the genotypes. According to Rahman *et al.* (2004) all the varieties culture with 3.0-4.0 mg/l of 2,4-D showed best performance on the callus induction on modified MS media.

The interaction effects of variety and callus media have significant effects on days of callus initiation. The lowest number of days (24.67) required for callus initiation

Table 14 : Callus initiation potentiality of three sugarcane varieties under different auxin (NAA and 2,4-D) concentrations

Variety	Days to callus initiation	Explants induced callus (%)
Isd 16	18.87	43.56
Isd 36	18.97	44.44
Isd 37	18.53	45.10
LSD (0.05)	0.272	1.494

Table – 15 : Effects of auxin (NAA and 2,4-D) in MS medium on callus induction from leaf sheath explants of sugarcane

Auxin (mg/l)	Days to callus initiation	Explants induced callus (%)
NAA		
1.0	0.00	0.00
2.0	27.89	15.55
3.0	28.22	13.30
4.0	0.00	0.00
5.0	0.00	0.00
2,4-D		
1.0	27.44	74.07
2.0	25.56	82.96
3.0	25.78	88.89
4.0	25.78	92.59
5.0	27.22	76.30
LSD (0.05)	0.497	2.729

Table 16. Interaction effects of varieties and auxin (NAA and 2,4-D) concentrations on callus formation in sugarcane

Variety X Auxin (mg/l)	Days to callus initiation	Explant induced callus (%)
Isd 16 X NAA 1.0	0.00	0.00
X NAA 2.0	26.67	11.11
X NAA 3.0	28.33	20.00
X NAA 4.0	0.00	0.00
X NAA 5.0	0.00	0.00
Isd 36 X NAA 1.0	0.00	0.00
X NAA 2.0	28.67	15.55
X NAA 3.0	27.67	889
X NAA 4.0	0.00	0.00
X NAA 5.0	0.00	0.00
Isd 37 X NAA 1.0	0.00	0.00
X NAA 2.0	28.33	20.00
X NAA 3.0	28.67	11.00
X NAA 4.0	0.00	0.00
X NAA 5.0	0.00	0.00
Isd 16 X 2,4-D 1.0	28.33	71.11
X 2,4-D 2.0	26.33	84.45
X 2,4-D 3.0	26.00	95.55
X 2,4-D 4.0	25.67	82.22
X 2,4-D 5.0	27.33	71.11
Isd 36 X 2,4-D 1.0	28.33	75.55
X 2,4-D 2.0	25.67	82.22
X 2,4-D 3.0	25.33	86.67
X 2,4-D 4.0	26.33	97.78
X 2,4-D 5.0	27.67	77.78
Isd 37 X 2,4-D 1.0	25.67	75.55
X 2,4-D 2.0	24.67	82.22
X 2,4-D 3.0	26.00	84.45
X 2,4-D 4.0	25.33	97.78
X 2,4-D 5.0	26.67	80.00
LSD (0.05)	0.861	4.726

in the variety Isd37 on medium contained 2, 4-D 2.0 mg/l (Table-16). The highest number of days (28.33) required for callus initiation in variety Isd16 and Isd36 on MS medium supplemented with 2, 4-D 1.0 mg/l. Among the interactions Isd37 in different concentrations of 2, 4-D showed better results than that of other interactions. The present experiment indicated that for callus induction different varieties required of different amount of 2, 4-D concentrations.

Similar observation was reported by many scientists who found that MS medium supplemented with 2, 4-D was the best for callus induction as in monocotyledons and even in dicotyledons (Evans *et al.* 1989; Jaisal and Narayan. 1985, Begum *et al.* 1995, Nadir *et al.* 1978, Liu 1984, Chen *et al.* 1998 and Lal and Singh 1991*)*. In this investigation, the best callus was observed at 4.0 mg/l of 2,4-D for all the varieties. These findings agreed with the findings of Begum *et al.*,(1995) for callus initiation using MS medium supplemented with 3.0-4.0 mg/l of 2,4-D in sugarcane varieties of Bangladesh. Similar response was reported by Barba *et al.* (1977) and Mannan and Amin (1999).

4.3.4. Effects on percentage of explants showed callus induction

The effects of varieties and auxin (NAA and 2,4-D) concentrations and their interaction on percentage of callus initiation was presented in the Table 14-16. The highest percentage of callus initiation (45.10%) was observed in the variety Isd37 and the variety Isd16 produced the lowest percentage (43.56%) of callus (Tabl-14). Variety Isd36 showed similar result and the percentage of callus initiation were (44.44%).

The percentage of callus initiation was also affected by different concentration of auxins (NAA and 2,4-D) supplemented into medium. Among different concentrations of NAA and 2,4-D ,the results of percentage of callus initiation with NAA were very lower than 2,4-D concentrations. So, we here discuss about affects of 2,4-D concentration.

The percentage of callus initiation was affected by different concentration of 2,4-D. The highest percentage (92.59%) of callus initiation was observed in the media supplemented with 4.0 mg/l of 2,4-D (Table-15). The lowest value (74.07%) was observed in MS medium supplemented with 1.0 mg/l of 2,4-D. The results were

agreed with the results of Hossain *et al* (1996) who found that the highest concentration produced the highest percentage of callus.

Interaction effects of varieties and 2,4-D concentrations on percentage of callus initiation had remarkable effects. However the variety Isd36 and Isd37 showed that 97.78% explants induced callus on 4.0 mg/l 2,4-D (Table-16).

The results of the present investigation were agreed with the results of Hossain *et al.* (1996) who found that the highest concentration produced the highest percentage of callus. Begum *et al.* (1995) found 3-5 mg/l of 2,4-D produced highest percentage of callus in Bangladeshi sugarcane varieties (viz. L. Jaba, Isd16, Isd20 and Clone 1/123)

4.3.5. Effects on Shoot proliferation from callus

The calli induced from callus media 2,4-D of three varieties of sugarcane were transferred to nine different types of concentrations and combinations of media for differentiation and proliferation of shoots. It was observed that the number of shoot production per culture, the number of usable shoot per culture and shoot length differed significantly among varieties and shooting media. Days to shoot initiation did not differ significantly. Data are presented on Tables 17-19; and Plate no. 3.2.

4.3.6. Effects on days of shoot initiation (DSI)

For days of shoot initiation among the factors varietal effect was not significant and shoot media was significant. The effect of varieties on days to shoot initiation was found not significant. The shoot initiation days required for variety Isd37 was the lowest (26.22) and the highest days (26.46) required to shoot initiation was Isd36 and the variety Isd16 was showed (26.35) statistically identical result (Table-17).

The effects of shooting media were significant for days to shoot initiation. The lowest days (25.11) required for shoot initiation was in the MS medium supplement with $BA_{1.0}NA_{0.2}$ (Table-18) This result was significantly higher over that of other treatments. The highest days (27.44) required for shoot initiation was in the MS medium supplement with $BA_{2.0}NA_{0.2}$. These results indicated that $BA_{1.0}NA_{0.2}$ was the best performance for days to shoot initiation.

Combined effects of variety and shoot media on production of shoot for days to shoot initiation were presented on the Table 19. The variety Isd37 were produced

shoot within the lowest days (24.67) on shooting media $BA_{1.0}$ $NA_{0.2}$containing MS medium. The highest days (27.67) required for the shoot initiation was in the variety Isd36 and MS medium supplemented with $BA_{0.5}IB_{0.5}$. In this experiment, Isd37 and $BA_{1.0}$ $NA_{0.2}$ was the best combination for shoot initiation.

4.3.7. Effects on number of shoot per culture

No significant result was obtained for shoot per culture incase of the main effect of variety on production of shoot from the Table 17; the highest number of shoot (7.49) was observed in the variety Isd37. Lowest number of shoots (7.22) was found in the variety Isd36 followed by the variety Isd16 with statistically identical result. So, the variety Isd37 showed effective performance for shoot production. This value was not significant over that of other varieties.

Highly significant result was observed for shoot per culture incase of shooting media on shoot production. The MS media supplemented with $BA_{1.0}$ $NA_{0.5}$ which produced the highest number of shoots (13.23) per culture (Table-18). Lowest performance (4.78) was observed with $BA_{0.5}IB_{0.5}$ containing MS medium. Here MS medium supplemented with $BA_{1.0}$ $NA_{0.5}$ was the best performing treatment.

Significant result was found in the combined effect of variety and shooting media for shoot per culture. Isd16 produced highest number (13.80) of shoots per culture in the MS medium supplemented with $BA_{1.0}NA_{0.5}$. (Table-19) The lowest number of shoot (4.43) was observed in the variety Isd16 with shooting medium $BA_{2.0}NA_{0.2}$. From this investigation Isd16 and MS medium supplemented with $BA_{1.0}NA_{0.5}$ showed the best performance among the variety and shooting media respectively.

4.3.8. Effects on number of usable shoots per culture

The number of usable shoots is important for in intro plantlet production. Effects of variety, effects of shooting media and interaction effects of variety and shooting media exert significant effects on number of usable shoots per culture.

The main effect of varieties for usable shoot per culture showed significant result from the Table 17; the highest number of usable shoot (3.90) was observed in the variety Isd37. Lowest number of shoots (3.81) was found in the variety Isd36 followed by the variety Isd16 with statistically identical result. So, the variety Isd 37

Table 17. Effects of three sugarcane varieties on shoot production from callus tissue under *in vitro* condition

Variety	Days to shoot initiation	No. of total shoot per culture	No. of usable shoot per culture	Shoot length (cm)
Isd 16	26.35	7.30	3.83	3.66
Isd 36	26.46	7.22	3.81	3.62
Isd 37	26.22	7.49	3.90	3.75
LSD	0.257	0.304	0.068	0.040

Table 18 : Effects of the cytokinine (BA) with auxin (NAA or IBA) at different concentrations and combinations in MS medium on shoot production from the callus tissue of three sugarcane varieties

Combination & Concentration of growth regulators (mg/l)	Days to shoot initiation	No. of total shoot per culture	No. of usable shoot per culture	Shoot length (cm)
$B_{0.5}$ $NA_{0.1}$	25.33	6.80	3.44	3.51
$B_{0.5}$ $NA_{0.2}$	26.78	7.50	3.83	3.86
$B_{0.5}$ $NA_{0.5}$	26.67	8.00	4.20	4.18
$B_{1.0}$ $NA_{0.1}$	26.89	9.16	4.70	4.38
$B_{1.0}$ $NA_{0.2}$	25.11	10.11	5.41	4.92
$B_{1.0}$ $NA_{0.5}$	26.33	13.23	6.02	5.18
$B_{2.0}$ $NA_{0.1}$	26.11	5.96	3.54	2.97
$B_{2.0}$ $NA_{0.2}$	27.44	4.94	2.86	2.51
$B_{2.0}$ $NA_{0.5}$	26.67	4.87	2.71	2.39
$B_{0.5}$ $IB_{0.1}$	25.22	5.40	2.71	2.52
$B_{0.5}$ $IB_{0.2}$	27.00	6.83	3.67	3.23
$B_{0.5}$ $IB_{0.5}$	25.89	4.78	2.99	2.83
$B_{1.0}$ $IB_{0.1}$	25.89	6.44	3.60	3.74
$B_{1.0}$ $IB_{0.2}$	26.67	8.10	4.52	3.87
$B_{1.0}$ $IB_{0.5}$	27.11	10.86	5.30	4.54
$B_{2.0}$ $IB_{0.1}$	26.56	6.13	2.93	3.87
$B_{2.0}$ $IB_{0.2}$	27.00	7.21	3.96	4.24
$B_{2.0}$ $IB_{0.5}$	25.56	5.73	2.84	3.42
LSD (0.05)	0.631	0.746	0.167	0.098

Table 19 : Interaction effects between varieties, shooting media on shoot production from callus tissue of sugarcane.

Variety X Shooting media	Days to shoot initiation	No. of total shoot per culture	No. of usable shoot per culture	Shoot length (cm)
lsd 16 X $B_{0.5}$ $NA_{0.1}$	25.67	6.43	3.43	3.50
$B_{0.5}$ $NA_{0.2}$	27.33	6.57	3.53	4.00
$B_{0.5}$ $NA_{0.5}$	26.67	7.23	3.93	4.53
$B_{1.0}$ $NA_{0.1}$	27.00	8.33	4.63	4.30
$B_{1.0}$ $NA_{0.2}$	25.33	10.60	5.63	4.90
$B_{1.0}$ $NA_{0.5}$	27.33	13.80	6.07	5.13
$B_{2.0}$ $NA_{0.1}$	26.67	5.80	3.50	3.17
$B_{2.0}$ $NA_{0.2}$	27.33	4.43	2.73	2.53
$B_{2.0}$ $NA_{0.5}$	26.00	4.83	2.90	2.30
$B_{0.5}$ $IB_{0.1}$	24.67	5.27	2.63	2.17
$B_{0.5}$ $IB_{0.2}$	26.67	6.57	3.50	3.17
$B_{0.5}$ $IB_{0.5}$	25.67	5.13	2.70	2.90
$B_{1.0}$ $IB_{0.1}$	25.67	7.30	3.87	3.13
$B_{1.0}$ $IB_{0.2}$	26.33	8.53	4.63	3.43
$B_{1.0}$ $IB_{0.5}$	27.33	11.43	5.37	4.70
$B_{2.0}$ $IB_{0.1}$	27.33	6.17	3.10	4.37
$B_{2.0}$ $IB_{0.2}$	26.33	7.53	4.27	4.10
$B_{2.0}$ $IB_{0.5}$	25.00	5.47	2.53	3.63
lsd 36 X $B_{0.5}$ $NA_{0.1}$	24.67	6.70	3.50	3.50
$B_{0.5}$ $NA_{0.2}$	26.33	7.73	3.83	3.90
$B_{0.5}$ $NA_{0.5}$	26.67	8.17	4.10	4.23
$B_{1.0}$ $NA_{0.1}$	26.67	9.73	4.83	4.43
$B_{1.0}$ $NA_{0.2}$	25.33	8.00	5.13	4.97
$B_{1.0}$ $NA_{0.5}$	25.67	12.73	5.77	5.27
$B_{2.0}$ $NA_{0.1}$	26.00	5.93	3.57	2.63
$B_{2.0}$ $NA_{0.2}$	27.33	4.80	2.87	2.47
$B_{2.0}$ $NA_{0.5}$	27.00	4.77	2.63	2.23

Contd. (Table 19)

Variety X Shooting media	Days to shoot initiation	No. of total shoot per culture	No. of usable shoot per culture	Shoot length (cm)
Isd 36 X $B_{0.5}$ $IB_{0.1}$	25.33	5.30	2.77	2.20
$B_{0.5}$ $IB_{0.2}$	27.67	6.97	3.53	3,07
$B_{0.5}$ $IB_{0.5}$	27.67	4.50	3.13	3.00
$B_{1.0}$ $IB_{0.1}$	25.67	6.27	3.33	4.03
$B_{1.0}$ $IB_{0.2}$	27.00	8.17	4.43	3.63
$B_{1.0}$ $IB_{0.5}$	27.33	10.50	5.27	3.83
$B_{2.0}$ $IB_{0.1}$	27.33	6.10	2.73	4.10
$B_{2.0}$ $IB_{0.2}$	27.33	7.03	4.03	4.50
$B_{2.0}$ $IB_{0.5}$	26.33	6.53	3.17	3.10
Isd 37 X $B_{0.5}$ $NA_{0.1}$	25.67	7.27	3.40	3.53
$B_{0.5}$ $NA_{0.2}$	26.67	8.20	4.13	3.67
$B_{0.5}$ $NA_{0.5}$	26.67	8.60	4.57	3.77
$B_{1.0}$ $NA_{0.1}$	27.00	9.40	4.63	4.40
$B_{1.0}$ $NA_{0.2}$	24.67	11.73	5.47	4.90
$B_{1.0}$ $NA_{0.5}$	26.00	13.17	6.23	5.13
$B_{2.0}$ $NA_{0.1}$	25.67	8.13	3.57	3.10
$B_{2.0}$ $NA_{0.2}$	27.67	5.60	2.97	2.53
$B_{2.0}$ $NA_{0.5}$	27.00	5.00	2.60	2.63
$B_{0.5}$ $IB_{0.1}$	25.67	5.63	2.73	3.20
$B_{0.5}$ $IB_{0.2}$	26.67	6.97	3.97	3.47
$B_{0.5}$ $IB_{0.5}$	25.33	4.70	3.13	2.60
$B_{1.0}$ $IB_{0.1}$	26.33	5.77	3.60	4.07
$B_{1.0}$ $IB_{0.2}$	26.67	7.60	4.50	4.53
$B_{1.0}$ $IB_{0.5}$	26.67	10.63	5.27	5.10
$B_{2.0}$ $IB_{0.1}$	25.00	6.13	2.97	3.13
$B_{2.0}$ $IB_{0.2}$	27.33	7.07	3.57	4.13
$B_{2.0}$ $IB_{0.5}$	25.33	5.20	2.83	3.53
LSD (0.05)	1.094	1.293	0.289	0.169

showed effective performance for production of usable shoot. This value was significant over that of other varieties.

Highly significant result was observed for usable shoot per culture incase of shooting media on production of usable shoot. The MS media supplemented with $BA_{1.0}$ $NA_{0.5}$ which produced the highest number of usable shoots (6.02) per culture (Table-18). Lowest performance (2.71) was observed with $BA_{2.0}IB_{0.1}$ or $BA_{2.0}NA_{0.5}$ containing MS medium. Here MS medium supplemented with $BA_{1.0}$ $NA_{0.5}$ was the best performing treatment.

Highly significant result was found in the combined effect of variety and shooting media for usable shoot per culture. Isd37 produced highest number (6.23) of shoots per culture in the MS medium supplemented with $BA_{1.0}NA_{0.5}$. The lowest number of shoot (2.53) was observed in the variety Isd16 with shooting medium $BA_{2.0}IB_{0.5}$. From this investigation Isd37 and MS medium supplemented with $BA_{1.0}NA_{0.5}$ showed the best performance among the variety and shooting media respectively.

4.3.9. Effects on shoot length

Shoot length is depends on varieties and shooting media. Effects of variety, effects of shooting media and interaction effects of variety and shooting media have remarkable effects on shoot length.

Highly significant result was obtained for shoot length in case of the main effect of variety. The variety Isd37 showed highest shoot length (3.75 cm) was significantly higher over other varieties, followed by the variety Isd16 with 3.67cm (Table-17). The lowest shoot length (3.6cm) was founded in the variety Isd36. All varieties gave the better shoot length. These results indicate that the variety Isd37 was the best for increasing shoot length.

In case of the main effect of shooting media was observed highly significant for the shoot length. The MS media supplemented with $BA_{1.0}NA_{0.5}$ which produced the highest shoot length (5.18cm) (Table-18). Lowest performance (2.39cm) was observed with $BA_{2.0}NA_{0.5}$ containing MS medium. Here MS medium supplemented with $BA_{1.0}NA_{0.5}$ was the best performing treatment.

Interaction effects of the variety and shoot media for shoot length were highly significant. The variety Isd36 showed highest shoot length (5.27cm) on shooting

media $BA_{1.0}NA_{0.5}$ containing MS medium (Table-19). The lowest shoot length (2.17cm) was found in the variety Isd16 and MS medium containing $BA_{0.5}IB_{0.1}$ alone. From this investigation Isd36 and MS medium supplemented with $BA_{1.0}NA_{0.5}$ showed the best performance among the variety and shooting media respectively.

The result of this study is consistent with the hypothesis of Skoog and Miller (1957). Similar response was also obtained by Alam *et al* (2003) for sugarcane variety Isd 18 at the combination of 1.0 mg/l BA + 0.5 mg/l NAA. The combination of 1.0 mg/l BA + 0.5 mg/l IBA also showed effective result for shoot multiplication. Karim *et al.* (2002) observed positive response in in terms o multiple shoot formation on MS medium supplemented with 1.0 mg/l BA + 0.5 mg/l IBA.

4.4. Production of roots in regenerated shoots

Root formation is very important on regenerated shoots for establishment of *in vitro* grown plantlets. Effects of auxins (NAA and IBA) on root formation in shoots regenerated from callus of leaf sheath explant of sugarcane varieties were investigated. Induction and development of roots at the base of the in vitro grown shoots is an indispensable step to establish micropropagation derived plantlets on the soil. For this purpose, in vitro grown proliferating micro shoots were isolated and cultured on MS medium supplemented with concentration of 0.5, 1.0, 3.0 and 5.0 mg/l NAA and IBA for root formation to establish in the field. Effects of root formation for varieties, NAA, IBA and their interactions are presented on the Tables 20-22. and Plate no. 4..

4.4.1. Effects on days of root initiation

The main effects of varieties on days to root initiation showed significant differences. The lowest days (14.04) required for root initiation in the variety Isd16 showed the best result than other varieties and followed by the variety Isd36 with statistically identical result (Table-20). But days to root initiation from Isd37 was not satisfactory. These findings were similar with the results of Naz, S. (2004) who observed that half strength MS medium with 5 mg/l NAA exhibited the high rate of root formation within 10-15 days.

The effects of auxins (NAA and IBA) had significant influences for days of root initiation. The lowest days (13.44) required for initiation of root from shoot in the

Isd16

Isd36

Isd37

Plate 4. Growth of roots of initiated shoots of Sugarcane under *in vitro* condition

MS medium supplemented with 5 mg/l NAA (Table-21). Higher concentration of NAA (5 mg/l) showed better result for days of root initiation than other concentration. So, the 5 mg/l NAA in MS medium is the best for root initiation.

The interaction effects of varieties and rooting media for days of root initiation were found significant result. The lowest days (12.33) required for root initiation was in the variety Isd16 on rooting media of 5.0 mg/l NAA and the highest days(16.67) required for root initiation was obtained from the variety Isd37 and MS media supplemented with 3.0 mgl of NAA or IBA (Table-22). The interaction effect Isd16 and MS media supplemented with 5.0 mg/l NAA exhibited best result than that of other treatments.

The present findings agreed with the report of Karim *et al.* (2002), who found that higher doses of auxin especially NAA is required for efficient root development in sugarcane. Lal (1992) and Rongrong *et al.* (2002) also reported that NAA is most suitable auxin for rooting particularly in sugarcane.

4.4.2. Root per shoot

Incase of root per shoot, the highest number of roots (9.68) per shoot was obtained from the variety Isd37 and the lowest number of roots (8.53) per shoot was found in the variety Isd36 (Table-20). The rate of root per shoot (9.17) in the variety isd16. So, the production of root per shoot, Isd37 was presented best result (9.68) this value was significantly higher than other varieties.

Number of roots per shoot was also affected by different concentrations of auxin (NAA and IBA) containing MS medium. The highest number of roots per shoot (12.94) was found at 5.0 mg/l of NAA which significantly higher over other treatments. Lower to medium concentration of auxin (NAA and IBA) gradually inhibit the number of roots per shoot (Table -21). According to Bakhsha *et al.* (2002) who found that half MS medium supplemented with NAA or IBA (5.0 mg/l) was suitable for roots and production of root from shoot.

The interaction effect of variety and rooting media showed highly significant result The highest number of roots per shoot(13.47) was obtained from the variety Isd37 and on rooting media 5.0 mg/l NAA (Table-22). The variety Isd36 and MS medium supplemented with 0.5 mg/l of IBA gave the lowest number of roots per shoot (4.27). Best interactions were media 5.0 mg/l NAA in this experiment. This result is

Table 20. Effects of varieties on root production cultured on MS media with various concentrations of auxin (NAA and IBA)

Variety	Days to root initiation	No. of root per shoot	Root length (cm)
Isd 16	14.04	9.17	1.53
Isd 36	14.13	8.53	1.52
Isd 37	15.21	9.68	1.60
LSD (0.05)	0.507	0.082	0.041

Table 21. Effect of auxin (NAA and IBA) concentrations in MS medium on root production in sugarcane

Auxin (mg/l)	Days to root initiation	No. of root per shoot	Root length (cm)
NAA 0.5	13.89	5.27	1.51
NAA 1.0	14.78	8.84	2.07
NAA 3.0	15.44	11.53	1.91
NAA 5.0	13.44	12.94	1.51
IBA 0.5	13.89	4.46	1.36
IBA 1.0	14.67	8.73	1.51
IBA 3.0	14.89	10.24	1.44
IBA 5.0	14.67	10.97	1.10
LSD (0.05)	0.828	0.134	0.067

Table 22. Interactions between varieties and rooting media on root production

Variety X Media	Days to root initiation	No. of roots per shoot	Root length (cm)
Isd 16 X NAA 0.5	13.33	5.17	1.43
X NAA 1.0	14.00	8.47	2.03
X NAA 3.0	15.67	11.53	1.83
X NAA 5.0	12.33	12.83	1.53
X IBA 0.5	14.33	4.53	1.37
X IBA 1.0	15.67	9.30	1.57
X IBA 3.0	13.33	10.53	1.37
X IBA 5.0	13.67	10.97	1.13
Isd 36 X NAA 0.5	13.67	5.07	1.47
X NAA 1.0	14.67	8.10	2.03
X NAA 3.0	14.00	10.90	1.93
X NAA 5.0	13.67	12.53	1.40
X IBA 0.5	14.00	4.27	1.37
X IBA 1.0	13.33	7.27	1.53
X IBA 3.0	14.67	9.53	1.37
X IBA 5.0	15.00	10.57	1.03
Isd 37 X NAA 0.5	14.67	5.57	1.63
X NAA 1.0	15.67	9.97	2.13
X NAA 3.0	16.67	12.17	1.97
X NAA 5.0	14.33	13.47	1.60
X IBA 0.5	13.33	4.57	1.33
X IBA 1.0	15.00	9.63	1.43
X IBA 3.0	16.67	10.67	1.60
X IBA 5.0	15.33	11.37	1.13
LSD (0.05)	1.435	0.232	0.116

agreed with result of Chegalrayan *et al.* (2001) and Geetha *et al.* (2001) who observed that all varities showed better response on MS medium supplemented with 5.0 mg/l of NAA. The highest root per shoot was obtained in the variety Isd28 using NAA 5.0 mg/l was reported by Baksha *et al.* (2002).

4.4.3. Root length

The effect of variety on root length was significant. Among the varieties, the highest root length (1.60cm) was founded in the variety Isd37 (Table-37). This variety gave best result from other varieties .But the variety Isd36 showed the lowest root length (1.52cm) and followed by the variety Isd16 with statistically identical result.

In case of the main effect of rooting media, all the treatments showed significant variation in root length. With 1.0 mg/l of NAA, root length (2.07cm) was significantly higher over other treatments (Table-21). The lowest root length was found in 5.0 of IBA (Table -21). These result indicated that 1.0 mg/l of NAA was the best for increasing root length.

Interaction effects of the variety and rooting media for root length were highly significant. The highest root length (2.13cm) was obtained in the variety Isd37 in the concentration of 1.0 mg/l NAA containing MS medium (Table-22). The lowest performance (1.03cm) was found in the variety Isd36 and MS medium containing 5.0 mg/l of IBA. From this investigation Isd37 and MS medium supplemented with 1.0 mg/l NAA showed the best performance.

In this present investigating, it was observed that IBA was comparatively less effective than NAA for producing roots for all the varieties. No. of roots and length of the root is vice versa.

4.5. Establishment of the plantlets:

After rooting to the *in vitro* regenerated shoots they were transferred to soil before transplantation. The individual plantlets were taken out from the culture media and the roots of the plantlet were washed made gel free by continuous flowing for water. Then the plants were transferred on the sun dried soil containing sand in pots. The potted plants were then watered adequately, covered with perforated polyethylene bag and kept in the growth camber for 10-12 days. Among the transplanted plants 80-92% were survived and acclimatized successfully on the soil. The best of the

transplants could not survive under the *ex vitro* due to desiccation and microbial effects over growth. The survival of the plantlets under the *ex vitro* condition of the soil also influenced by state of root growth and general health of the plantlets. The survival rate of healthy plantlets have 1-2 cm. root length was more than those plantlets having elongated roots but not healthy.

In this regard best results were observed on the medium supplemented with 2.0 mg/l BA and 2.0 mg/l Kn. On this growth regulator combination 50% explant of the variety Isd16 and Isd37, 40% explants of the varieties Isd36, showed growth. On the other hand 30% explants of Isd16 and Isd37, 20% explants of Isd36 shoot growth with 2.0 mg/l of Kn. However, after 5 weeks of culture on the medium containing different concentrations of cytokinins (BA or Kn) but no auxin the cultured explant showed only enlargement. Except elongation and enlargement of the explant no proliferation of the axillary shoots was observed in the media supplemented with different concentrations of the cytokinin alone. Results of this experiment are summarized and presented on the Table-2.

Chapter V
Summary and Conclusion

CHAPTER V

SUMMARY AND CONCLUSIN

The present study was carried out to develop a suitable reproducible protocol for micropropagation of BSRI released three sugarcane varieties viz. Isd16, Isd36 and Isd37. With this view, the experiment was conducted in the Germplasm Laboratory of the Breeding division, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, during the period from August 2007 to May 2008. The experiment was conducted following Completely Randomized Design (CRD). Bud, shoot tip and leaf sheath were used as explants.

Surface sterilization of the explants from the field grown plants was done with 0.1% $HgCl_2$ for 10 minutes.

Incase of direct regeneration, bud explants were cultured on MS medium with different concentrations and combinations of growth regulators. The explants cultured with only cytokinin (BA or Kn) failed to give satisfactory proliferation and elongation of auxillary shoots. On the other hand, the explants cultured with both cytokinin and auxin combinations ($BA_{2.0}NA_{0.2}$) showed better performance for all varieties. In shoot induction from the shoot tip explants, effectiveness of the combination of cytokinin (BAP) with auxins (NAA or IBA) was proved to be superior to that of concentration of cytokinin (BAP) alone. Among the concentrations of single cytokinin, 2.0 mg/l of BAP produced comparatively higher number of shoots. 2.0 mg/l of BAP in combination with different auxin concentrations (0.1, 0.2, 0.5 and 1.0 mg/l of NAA or IBA) were tested for shoot proliferation. Among the varieties, Isd37 showed the best performance for shoot initiation when media were supplemented with BAP 2.0 mg/l + NAA 0.2 mg/l.

Incase of indirect regeneration from the leaf sheath explants, callus induction was observed after 10-15 days of culture and calli grew vigorously until the nutrient of the medium exhausted. Among different concentrations of growth regulator 2,4-D is more effective than NAA for callus initiation. Best callus induction (97.78%) was observed in medium containing 4.0 mg/l of 2,4-D for the varieties Isd36 & Isd37

and 3.0 mg/l of 2,4-D for the variety Isd16. Callus induction percentage was increased with the increase of the concentration of 2,4-D but after a certain level (4.0 mg/l), this become decreased. It was observed that number of shoots per culture, useable shoots per culture and shoot length differed significantly among the varieties. The variety Isd37 showed best performance for shoot production followed by the variety Isd16 and Isd36 . The medium containing 1.0 mg/l BA + 0.5 mg/l NAA was the best in producing shoot. The highest number of shoots per culture(13.80) were obtained from the variety Isd16 on the medium containing 1.0 mg/l BA + 0.5 mg/l NAA.

The varieties differed significantly for production of root per shoot and root length. The highest number of roots per shoot (9.68) and the maximum root length (1.60 cm) was found in the variety Isd37. In the media effect, for root initiation NAA is more effective than IBA. 5.0 mg/l of NAA containing medium showed the best performance for all varieties. The highest number of roots per shoot (13.47) was found in the variety Isd37 followed by Isd16 on the medium supplemented with 5.0 mg/l of NAA.

The following conclusion could be drawn from the present investigation :

1. $BA_{2.0}NA_{0.2}$ supplemented MS medium could be used for shoot initiation for direct regeneration from the bud and shoot tip explants.
2. MS medium supplemented with 4.0 mg/l 2,4-D may be suggested for vigorous and high frequency callus initiation from the leaf sheath explants.
3. $BA_{1.0}NA_{0.5}$ containing MS medium was found suitable for shoot initiation from callus tissue and high frequency plantlet production.
4. 5.0 mg/l NAA containing MS medium may be recommended for growth and development of roots in shoots.
5. Leaf sheath was found as the most suitable explant for micropropagation of sugarcane.

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CHAPTER VI
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APPENDICES

Appendix 1: Analysis of data on shoot production from the bud explant under different Cytokinin (BA and Kn) concentration

Source of variation	Degrees of freedom	Mean square values		
		Days to Shoot Initiation	No. of shoot	Shoot length (cm)
Factor A (hormone)	1	7.511*	0.187**	1.878**
Factor B (Concentration)	4	16.461**	0.226**	1.227**
Factor AB	4	1.206ns	0.010**	0.013ns
Factor C (Variety)	2	0.478ns	0.018**	0.881**
Factor AC	2	0.544ns	0.001ns	0.117**
Factor BC	8	0.686ns	0.001ns	0.009ns
Factor ABC	8	0.364ns	0.002ns	0.013ns
Error	60	1.456	0.002	0.019

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant

Appendix 2: Analysis of data on shoot production from the bud explant under different combination of Cytokinin and Auxin.

Source of variation	Degrees of freedom	Mean square values		
		Days to Shoot Initiation	No. of shoot	Shoot length (cm)
Factor A (hormone)	1	0.000ns	0.190**	1.934**
Factor B (Concentration)	3	19.278**	0.287**	1.369**
Factor AB	3	0.407ns	0.009*	0.068**
Factor C (Variety)	2	1.056ns	0.015**	0.022ns
Factor AC	2	0.667ns	0.004ns	0.051*
Factor BC	6	0.444ns	0.001ns	0.030*
Factor ABC	6	0.574ns	0.002ns	0.040**
Error	48	0.736	0.002	0.010

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant

Appendix 3: Analysis of data on shoot production from the shoot tip explant under different Cytokinin (BA and Kn) concentration

Source of variation	Degrees of freedom	Mean square values		
		Days to Shoot Initiation	No. of shoot	Shoot length (cm)
Factor A (hormone)	1	1.344ns	11.378**	1.469**
Factor B (Concentration)	4	8.956**	4.289**	4.150**
Factor AB	4	1.067ns	0.078**	0.265**
Factor C (Variety)	2	1.011ns	0.294**	0.172**
Factor AC	2	1.344ns	0.108**	0.283**
Factor BC	8	2.289**	0.095**	0.095**
Factor ABC	8	0.317ns	0.010ns	0.110**
Error	60	0.567	0.010	0.009

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant

Appendix 4: Analysis of data on shoot production from the shoot tip explant under different combination of Cytokinin and Auxin

Source of variation	Degrees of freedom	Mean square values		
		Days to Shoot Initiation	No. of shoot	Soot length (cm)
Factor A (hormone)	1	0.000	41.102**	17.503**
Factor B (Concentration)	3	19.796**	4.043**	2.248**
Factor AB	3	0.333ns	0.469**	0.088*
Factor C (Variety)	2	0.681ns	0.794**	0.380**
Factor AC	2	0.292ns	0.062*	0.084ns
Factor BC	6	0.310ns	0.008ns	0.013ns
Factor ABC	6	0.069ns	0.004ns	0.018ns
Error	48	0.292	0.016	0.027

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant

Appendix 5: Analysis of data on callus initiation from the leaf sheath explant under different Auxin (NAA and 2,4-D) concentration

Source of variation	Degrees of freedom	Mean square values	
		Days to Callus Initiation	% of Callus
Factor A (hormone)	1	5152.900**	13407.925**
Factor B (Concentration)	4	979.428**	743.948**
Factor AB	4	1153.206**	389.190**
Factor C (Variety)	2	1.544**	18.031ns
Factor AC	2	4.433**	39.116*
Factor BC	8	1.211**	90.798**
Factor ABC	8	1156**	44.447**
Error	60	0.278	8.373

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant

Appendix 6: Analysis of data on shoot production from the Callus tissue under different combination of Cytokinin and Auxin

Source of variation	Degrees of freedom	Mean square values			
		Days to Shoot Initiation	No. of shoot	Usable shoot/ culture	Soot length (cm)
Factor A (hormone)	1	0.099ns	41.203**	8.820**	1.298**
Factor B (Concentration)	8	5.886**	79.573**	15.771**	7.425**
Factor AB	8	3.543**	14.962**	2.360**	5.447**
Factor C (Variety)	2	0.784ns	1.035ns	0.103*	0.232**
Factor AC	2	3.932**	5.271**	0.080ns	0.381**
Factor BC	16	1.353**	1.132*	0164**	0.414**
Factor ABC	16	0.418ns	1.126ns	0.135**	0.390**
Error	108	0.457	0.638	0.032	0.011

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant

Appendix 7: Analysis of data on root production from the initiated shoot under different Auxin (NAA and IBA) concentration

Source of variation	Degrees of freedom	Mean square values		
		Days to root Initiation	No. of root	root length (cm)
Factor A (hormone)	1	0.347ns	19.740**	2.840**
Factor B (Concentration)	3	6.347**	176.502**	0.881**
Factor AB	3	2.606*	2.785**	0.133**
Factor C (Variety)	2	10.167**	7.911**	0.052**
Factor AC	2	0.722ns	0.652**	0.018*
Factor BC	6	2.722**	0.828**	0.017**
Factor ABC	6	2.981**	0.206**	0.016**
Error	48	0.764	0.020	0.005

* = Significant at 5% level, ** = Highly Significant at 1% level, NS = Not Significant