IN VITRO REGENERATION STUDIES OF SUGARCANE

A

DISSERTATION

By

Ms. Milli Sharma

Roll No. 3030124

Submitted in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology

Department of Biotechnology and Environmental Sciences
Thapar Institute of Engineering and Technology
Patiala – 147004, India.

JUNE 2005

CERTIFICATE

This is to certify that the thesis entitled “In vitro regeneration studies of sugarcane.” submitted by Ms. Milli Sharma (3030124) in partial fulfillment of the requirements for the award of Master of Science in Biotechnology to Thapar Institute of Engineering and Technology Patiala, is a record of student’s own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any degree or certificate in this or any other university or institute.

(Mr. Dipal Roy Choudhury)
Supervisor
Research Scientist,
TIFAC, CORE
Thapar institute of Engg. And Tech
Patiala

Head
Deptt. Of Biotech. & Env. Sciences
Thapar institute of Engg. and Tech.
Patiala

Dean (Academic Affairs)
Thapar Institute of Engineering and Technology
Patiala

Abstract

Mass propagation protocol, callus induction and subsequent plant regeneration *in vitro* in Sugarcane cultivars, CoS 8436 and CoJ 83 were worked out. Axillary bud induction observed best (80-95%) from field grown (8-12 months old) shoot tip spindle explants within 7-10 days in semi solid MS media supplemented with BAP (0.5 mg/l) and Kn (0.5mg/l), however maximum shoots/explant (4.25 - 5.75) were observed in MS + BAP (1.5mg/l) + Kn (1.5mg/l). After two cycle serial subculture in semi solid media for establishment, shoot clumps were multiplied in liquid media, provide higher multiplication rate (3.63-4) in MS + BAP (1.0mg/l) + Kn (1.5mg/l) and higher shoot elongation in MS + BAP (0.5mg/l) + Kn (0.5mg/l). *In vitro* grown shoots/shoot clumps show earlier root induction (5-7 days), higher no of roots and root length in MS+ NAA (5 mg/l) + sugar (70gm/l). Spindle explants show better callus induction response in MS + 2, 4-D (3mg/l) within 14 days in dark and multiplied in reduced/half conc. of auxin. Plantlet regeneration (68-74%) were best obtained from compact nodular, morphogenic calli cultured in MS media supplemented with BAP (0.5mg/l) and Kn (0.5mg/l) cultured in light and show similar responses for shoot multiplication, elongation and rooting.

Acknowledgement

The day with its own brightness have finally come, when I can record my acknowledgements to all people who have been instrumental in shaping this manuscript.

To me, it is a proud and privilege to ventilate deep sense of gratitude and acclamation to my guide and mentor Mr. Dipal Roy Choudhury, Research Scientist, TIFAC, CORE, TIET, for his advice, supervision and encouragement. Without his guidance, constructive criticism and meticulous scrutiny, this work would not have seen light of the day.

I would like to express my cordial and faithful gratitude to Dr. Niranjan Das, Head, Dept of Biotechnology and Environmental Sc., TIET, for his kindness, support and professional guidance and valuable advice in many different ways.

I am also thankful to Prof. Sunil Khanna, Co-ordinator, TIFAC CORE and former Head, DBTES, TIET, for his guidance and immense concern throughout the project work.

My fervent thanks to Dr. Anil Kumar and Dr. K.S. Barna, Research Scientist, TIFAC CORE, for their valuable suggestions at regular interval.

I also appreciate cheerful cooperation rendered by Mr. Diwakar Aggarwal, Research Scholar, TIFAC, CORE.

I am also thankful to all the staff members of Tissue culture department at TIFAC-CORE with special mention to Mr Khem Raj, Mr Rajesh, Mr Deepak, Mr Bhupinder, Mr Anil, Mr Prakash, Mr Deepak, for their prompt help at different stages of the study.

I would also express my cordial thanks to all the office staff and persons in relation to TIFAC CORE and Department of Biotechnology and Environmental sciences, T.I.E.T, for sincere help.

I express my sincere thanks to Prof S.S.Gosal, Dept. of Genetics, Plant Breeding and Biotechnology, PAU, for his immensely valuable advice and guidance during the course.

Thanks are also due to Ms. Neetu Sharma, my batch mate, for her kind co-operation and valuable suggestions during this period.

I should like this opportunity to express my heartfelt thanks to Ms. Navneet Jaj, Ms. Saloni Bansal, Ms. Uruvashi Jaiswal, Ms. Shivani Tayal for their invaluable smiles and friendship during the course.

Above all I owe all this to my parents whose hard work throughout life gave me this opportunity. I am also indebted to my brother for his love and warmth.

Date : (Milli sharma)

Place:

Table of Contents

1. Introduction

1. a Historical record

Sugarcane culture dates from antiquity and originated in what is now New Guinea in the South Pacific about 8000 years ago and spread to the nearby Solomon Islands, the New Hebrides and then to New Caledonia (Brandes, 1956). Its cultivation spread along human migration routes to Southeast Asia, India, and Polynesia. Barber, 1931, found the earliest mention of sugarcane in Indian writings of the period 1400-1000 B.C. Its Eastward spread has been traced to the migrations of the Pacific Islanders when it arrived in Hawaii between 600 and 1100 A.D.

Sugarcane was introduced in China from India around 800 B.C. Crude sugar was being produced by 400 B.C. and by 700 A.D., it was being commercially exploited by Indians in the Sub-continent. In 510 B.C., a Persian military expedition recorded finding sugarcane in that sub-continent, and later, Alexander the Great also found it there. The mountains and deserts of Afghanistan, Balauchistan, and eastern Persia served as natural barriers against the spread of cane to other areas for centuries. It eventually reached Persia in the sixth century. Sugarcane culture slowly spread Westward reaching Persia by 500 A.D. The next migration was started by the Prophet Mohammed who, a few years before his death in 632 A.D., began a Holy War for the conversion of the world to Islam. When his armies conquered Persia, they found sugarcane and adopted its cultivation, carrying it with them in their conquests, now calling it "the Persian Reed". The Arabs were responsible for much of its spread as they took it to Egypt in 641 A.D. during their conquests using their highly developed skills in agriculture and chemistry, the Egyptians developed clarification, crystallization, and refining processes. From there, sugarcane continued its Westward journey across Northern Africa reaching Morocco. Then, crossing the Mediterranean to Southern Spain by 755 A.D. and to Sicily in 950 A.D. The sugar industry in Spain was very successful, with about 30,000 ha of cane being cultivated by about 1150 A.D. Around 1420, the Portuguese introduced cane into Madeira, from where it soon reached the

Canary Islands, the Azores, and West Africa (Purseglove, 1979). Columbus transported sugar cane from the Canary Islands to Hispaniola (now the Dominican Republic) on his second voyage in 1493 (Deerr, 1949; Purseglove, 1979). The original word for sugar is probably the Sanskrit word for sugar is shakkara, the East Indian word for sugar was "shekar", in Arabic, it was "al zucar", adopted in Spanish as "azucar", French as "sucre", German as "Zucker" and in English as "sugar."

In 10th century Europe, sugar was considered a valuable medicine. Later, it was considered a rare spice and its price was as high as that of pepper, saffron and cinnamon. Sugar shaped a good deal of history of the New World. The first New World sugar cane mill began grinding in about 1516 in the Dominican Republic. Sugar production spread to Cuba, Jamaica, Puerto Rico, and the other Greater Antilles by the end of the 1500's (Hagelberg, 1985). The history of sugar in the West is an important subject for understanding the emerging industrial power of Europe in the 1700s and 1800s. Sugar cane was grown extensively in the Caribbean and still growing on some islands. In colonial times, sugar was a major product of the triangular trade of New World raw materials, European manufactures and African slaves. France found its sugar cane islands so valuable it effectively traded Canada to Britain for their return at the end of the Seven Years War. The Dutch similarly kept Suriname, a sugar colony in South America instead of seeking the return of the New Netherlands (New Amsterdam). Cuban sugar cane produced sugar which received price supports from and a guaranteed market in the USSR; the dissolution of that country forced the closure of most of Cuba's sugar industry. Sugar cane is still a large part of the economy in Barbados, the Dominican Republic, Guadeloupe, Jamaica, Grenada and other islands. Brazil is a major grower of sugar cane where it is used to produce sugar as well as to provide the alcohol used in making gasohol and biodiesel fuel.

1. b. Botanical characters

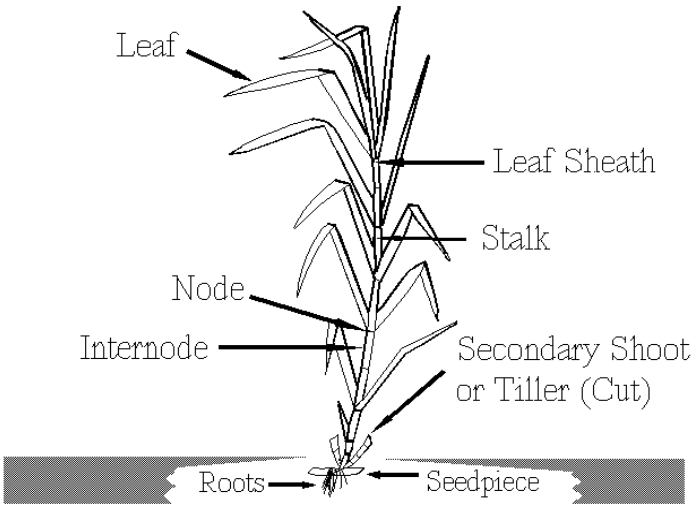
Sugarcane (*Saccharum officinarum L.)* 2n=80 to 205 is one of six species of a tall tropical southeast Asian grass (Family Poaceae) having stout fibrous

jointed stalks whose sap at one time was the primary source of sugar. Sugar cane is composed of six species of the genus *Saccharum,* in tribe Andropogoneae. All varieties of sugarcane are sp. or hybrids of the genus Saccharum. Species included are *S. officinarum, S. spontaneum, S. barberi, S. sinense, S. robustum,* and *S. edule*. There are two wild species, *S. spontaneum* and *S. robustum*, and 4 cultivated species, *S. officinarum,* S. *barberi*, *S. sinense*, and *S. edule* .The four cultivated species are complicated hybrids, and all intercross readily. All commercial canes grown today are inter-specific hybrids (Wrigley, 1982). It is a tall perennial tropical grass that tillers at the base to produce unbranched stems, 2.5-4 m or taller and about 3-5 cm in diameter. The solid unbranched stems, roughly circular or oval in cross section are clearly differentiated into joints, roughly circular or oval in cross section is clearly differentiated into joints, each comprising a node and an internode. A node consists of a lateral bud in leaf axils. Nodes are spaced at an interval of 0.15-0.25 m., but are much closer at the top of the stems (canes) of which the internodes vary in length (15-25 cm.), girth (1.5-6 cm in diameter) shape (cylindrical, conidial, barrel and circular or oval in cross section) colour (yellow, green, red, striped, variegated). Each stem has a hard, wax covered rind (epidermis), surrounding a mass of softer tissue parenchyma, which is interspersed with fibres.

MAIN PARTS OF THE PLANT

The main parts of the sugarcane plant are the stalk, leaf, and root system.

Figure 1. The sugarcane plant.



The Stalk

The stalk consists of segments called joints. Each joint is made up of a node and an internode (Figure 1 and Figure 2). The node is where the leaf attaches to the stalk and where the buds and root primordia are found. A leaf scar can be found at the node when the leaf drops off the plant. The length and diameter of the joints vary widely with different varieties and growing conditions. Generally the nodes are placed at an interval of 15-25 cm, but are much closer at the top of the stem, where elongation and rapid growth is taking place, than at the bottom, where they form part of the rootstock and are essential to the formation of tillers.

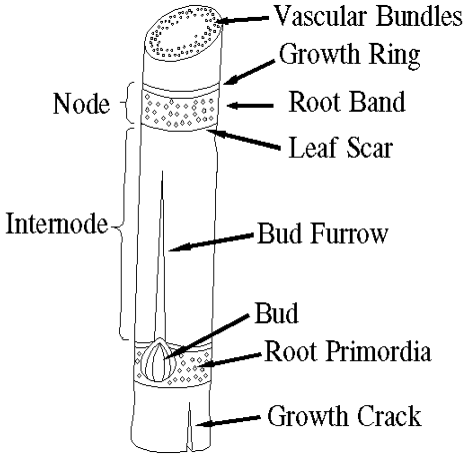


Figure 2. Parts of the stalk (stripped of leaves).

The buds, located in the root band of the node, are embryonic shoots consisting of a miniature stalk with small leaves (Figure 2). The outer small leaves are in the form of scales. The outermost bud scale has the form of a hood in commercial production, sugarcane is propagated from stem cuttings (setts, or seed pieces), each having two or more buds. Sugar accumulates in the stems (canes), of which the internodes (joints) vary in length (5-25 cm), girth (3-5 cm in diameter), shape (cylindrical, conoidal, barrel, or bobbin and circular or oval in cross-section), colour (yellow, green, red, purple, black, striped, variegated) and hardness according to growing conditions.

The Leaf

The leaf of the sugarcane plant is divided into two parts: sheath and blade, separated by a blade joint (Figure 3). The leaves are usually attached alternately to the nodes, thus forming two ranks on opposite sides. The mature sugarcane plant has an average total upper leaf surface of about 0.5 square meters and the number of green leaves per stalk is around ten, depending on variety and growing conditions.

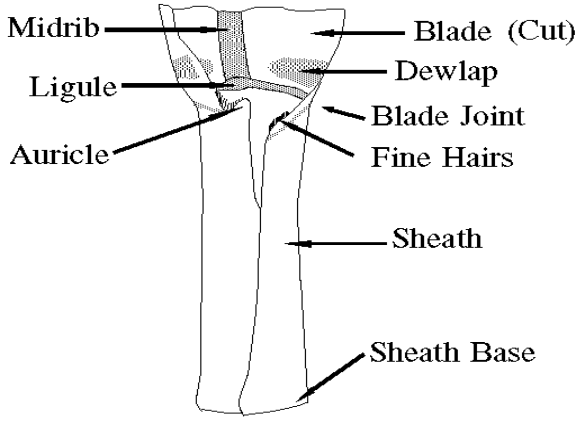


Figure 3. Parts of the leaf (peeled from the stalk).

The leaf has a strong midrib, white and concave on the upper surface, convex and green. The blade joint is where two wedge shaped areas called "dewlaps" are found (Figure 3). Leaf pubescence, or the covering of the various leaf parts with short hairs, is also variety and age dependent. Pubescence is not found on the leaf blade of commercial varieties, but does exist in sugarcane germplasm. Sheath pubescence can be used to identify plants.

The Inflorescence

The inflorescence, or tassle, of sugarcane is an open-branched panicle. Each tassle consists of several thousand tiny flowers, each capable of producing one seed. The seeds are extremely small and weigh approximately 250 per gram or 113,500 per pound. Generally, 12.5 hours day length and night temperatures between 20 -25° C induce floral initiation.

The Root System

The function of the root system is twofold: first, it enables the intake of water and nutrients from the soil; and second, it serves to anchor the plant. Two kinds of roots will develop from a planted seed piece. The set roots, which arise from the root band, are thin and highly branched; the shoot roots, originating from the lower root bands of the shoots, are thick, fleshy and less branched (Figure 4).

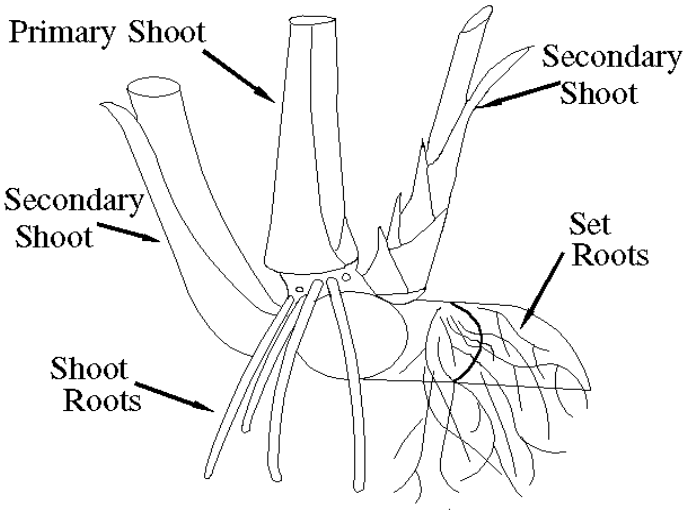


Figure 4. Set and shoot roots

1. c. Importance of sugarcane

Sugarcane accounts for approximately 70% of the world's sugar and is an economically important cash crop. Sugarcane contributes approximately 2% towards GDP of India. Major sugarcane producing states in India are Maharashtra and Uttar Pradesh, but sugarcane is also cultivated in Punjab, Haryana, Tamil Nadu, Karnataka, West Bengal and Bihar. Sugarcane in India is grown over an area of about 4.2 million. With over 450 sugar mills, India is the largest raw sugar producer in the world with capital employed totaling Rs.50, 000 crore. The sector provides direct employment to over 500,000 person while nearly 4.5 crore farmers are engaged in cane cultivation and

related activities. Over 11 million tons of refined sugar is produced, accounting for 60% of the total sugar cane cultivated.

Area under sugarcane, Yield per Hectare, Production (India)

Year	Area (Million hectares)	Production (Million ton)	Yield (kg / hectare)
1999-2000	4.02	299.2	70932
2000-2001	4.32	295.9	68577
2001-2002	4.40	301.0	68514
2002-2003	4.23	278.5	65870

Source: Agricultural statistics at a glance - 2003, http://www.agricoop.nic.in.

1. d Uses of Sugarcane

Sugar production and By-products

Sugarcane is an established agricultural field crop with a long history of safe use. Sugarcane is primarily grown as a source of sugar. Today, sugar cane has many industrial uses and is one of the most widely used and cheapest domestic products (Jenkins, 1966). Important industrial products of sugarcane include bagasse, molasses, and filter cake. Bagasse, the residual woody fiber of the cane, is used for several purposes: fuel for the boilers and lime kilns, production of numerous paper and paperboard products and reconstituted panel board, agricultural mulch, and as a raw material for production of chemicals. Bagasse and bagasse residues are primarily used as a fuel source for the boilers in the generation of process steam. Thus, bagasse is a renewable resource. Dried filter cake is used as an animal feed supplement, fertilizer, and source of sugarcane wax. Molasses is produced in two forms: inedible for humans (blackstrap) or as edible syrup. Blackstrap molasses is used primarily as an animal feed additive but also is used to produce ethanol, compressed yeast, citric acid, and rum. Edible molasses syrups are often blends with maple syrup, invert sugars, or corn syrup.

1. e Problems of sugarcane

Sale of sugar and Minimum support price

A higher MSP promotes sugarcane as a diversification crop and encourage farmers to break free from wheat-paddy cycle. Govt. of India announced a MSP for the 2002-03 crushing season. It is Rs 64.50 per quintal for a basic recovery rate of 8.5 per cent --- an increase of Rs 2.45 per quintal over last year. Then, a premium of Rs. 0.76 per quintal to be given for every 0.1 per cent increase in recovery. The total may thus go up to Rs 73 per quintal. After a prime ministerial announcement hiked the MSP by Rs. 5, to 69.50 the total may now reach Rs. 78 per quintal. But there are hurdles which discourage the farmers from taking to sugarcane in a big way and a remunerative MSP alone is not a sufficient attraction. Some of the state governments delay the announcement of “state advised prices”. According to press reports, sugar mills in Haryana owned Rs 100 crore to growers while the arrears in U.P amounted to a staggering Rs 1,000 crore. Since the income generated by sugarcane is only once a year, there is dire need to diversify the cropping system by introducing intercropping to generate mid season income for the farmers and additionally fulfilling the household requirements of food, besides mitigating the ill effects of sugarcane monoculture.

In December 2001, Government announced its policy to blend 5% ethanol in petrol in phased manner. Further by an announcement on 13 August 2002, 5% ethanol blended petrol has become mandatory from 1 January 2003 in 9 states and 4 union territories. This has surely given a boost to the sugar industry.

Diseases and Pests

There are many diseases and pests which may affect sugar cane. Bacterial diseases include gumming disease, *Xanthomonas vasculorum* (Cobb) Dows., in which yellowish stripes occur at the leaf tips and the vascular bundles exude a yellowish gum when cut, and leaf scald, *Xanthomonas albilineans* (Ashby) Dows., in which yellow stripes occur on the leaf blade, many side-

shoots are produced, and the vascular bundles of the stalk are red (Purseglove, 1979).

Fungal diseases such as red rot (*Colletotrichum falcatum* Went), root rot (*Pythium graminicolum* Subr.), pineapple disease (*Thielaviopsis parodoxa* (de Seynes) C. Moreau), downy mildew (*Sclerospora sacchari* Miy), and smut (*Ustilago scitaminea* Syd.) can also cause damage. Red rot causes the setts to be seriously damaged at low temperatures. Root rot was responsible for the failure of "Otaheite" (a noble cane) in Mauritius in 1846 and several other areas later. Pineapple disease attacks the setts causing the center to turn black and smell like overripe pineapples. Downy mildew is currently only found in the western Pacific and was responsible for severe losses in Queensland until rigorous controls were initiated. Smut causes black whiplike organs to emerge from the center of the leaf-roll and is important in southeastern Asia and South Africa (Purseglove, 1979).

Mosaic is a viral disease, whose vectors include *Aphis maidis* Fitch, was first recognized in Java in 1892 and causes severe stunting in some cases. Other viral diseases include ratoon stunting, chlorotic streak, Fiji disease, and Sereh disease (Purseglove 1979). The most destructive insects of sugar cane are stem-borers. Biological control, the use of natural parasites, is the most effective control for these (Purseglove 1979). Termites are also contributing to the loss of sugarcane in Indian. Rats are also a problem in many areas, for they eat the cane and introduce pathogens.

1. f Objectives of the present study

Micropropagation of sugarcane may be used to produce a large number of elite plantlets within a short time period. The present study was undertaken to establish a protocol for large-scale clonal propagation of variety CoS 8436, CoJ 83 through *in vitro* culture.

- To standardize in vitro mass propagation protocol of two commercially important varieties CoS 8436 and CoJ 83.
- To study nutritional aspects for callus culture maintenance and regeneration

Reliable proliferation of callus and subsequent plant regeneration are important for selection of sugarcane *in vitro*. The technique is also been used for genetic transformation studies undertaken for introduction of novel phenotypes, which is otherwise not possible to introduce through conventional breeding methods involving hybridization and subsequent selection or population improvement. Callus cultures are maintained and successful regeneration may produce plantlets having somaclonal variation (due to unorganized growth of cells) or callus cultures may be subjected to selection against abiotic/biotic stresses. This process may be helpful for *in vitro* selection and reduce the time needed in plant breeding programme.

2. Review of literature

2. a Mass propagation in vitro

2. a.1 Source of explant

Hendre *et al,* 1983, reported that about 2 lac plants can be produced in six months from a single shoot tip. *In vitro* micropropagation provides for the rapid multiplication of sugarcane. By this technology nearly 10,00,000 healthier, virus-free, type sugarcane plantlets can be produced from single plant in one year. It was also studied micro propagation of sugarcane using two procedures (1) shoot tip culture, (2) indirect somatic embryogenesis from callus and reported that Shoot tip culture produced plants phenotypically similar to the mother plant and gave a much more rapid multiplication rate compared to the other procedure (Lee *et al*, 1987)

Chen *et al,* 1988, reported sugarcane spindles of 6-12 months old plant were successful for shoot regeneration. Lal and Singh, 1994, reported use of different gelling agents like (agar and agarose), and support materials (filter paper bridge , cotton cloth bridge and adsorbent cotton) as well as shaken and static liquid (control) cultures to improve in vitro shoot multiplication and vigor in sugarcane. Dhumale *et al,* 1994, demonstrated that 2-3 mm shoot tips of sugarcane plant give significant shoot regeneration. Burner *et al*, 1995 propagated sugarcane in vitro from shoot tip.

Mullegadoo and Dookun, 1999, studied the explant source of sugarcane and it's effect on multiplication Explants from three sources, axillary bud, apical bud and shoot apex, were cultured. Severe bacterial contamination occurred in axillary buds resulting in necrosis and death of the explants. Growth responses were better with apical buds than with axillary buds in two of the three varieties cultured. By micropropagation over 1.5 million plants can be produced form a single shoot tip in six months (Anita *et al*, 2000). Ali and Afghan, 2001, reported micro propagation of sugarcane using meristem culture method and found that micropropagated plants were phenotypically similar to the mother plants. Noguera *et al*, 2002, described the

micropropagation of sugarcane from shoot apical. Hoy *et al,* 2003, studied the effect of tissue culture explant source on sugarcane yield components.

2. a. 2 Surface sterilization

Chen *et al*, 1998, has described surface sterilization of leaf explants with 95% ethanol for 5 minutes. Chengalrayan and Gallo-Meagher, 2001, reported surface sterilization of leaf explant by 0.5% sodium hypochlorite for 20 minutes. Mamun *et al*, 2004, reported that sterilization of leaf explant with 0.1% mercuric chloride for 8 to 10 min gave satisfactory results with, 85-90% of the explants being contamination free.

2. a. 3 Shoot multiplication

Chen *et al,* 1987, reported that at lower concentration of BAP (0.5 mg/l) and Kinetin (0.5 mg/l), there was loss of proliferation and vigour. Naritoom *et al*, 1993 reported plant regeneration from shoot tip culture of sugarcane using MS medium supplemented with cytokinin. Ali and Afghan, 2001, reported that basal medium (MS) supplemented with benzyl amine purine (BAP) and kinetin (Kn) gave rapid shoot multiplication. It was observed that at lower concentration of BAP, shoot proliferation was significant. Jose Carlos Lorenzo *et al,* 2001 showed a relationship between phenolic excretion and shoot formation. The most intensive period of phenolic excretion (11-20 days) preceded the most intensive period of shoot formation (21-30 days). Patel *et al*, 2001 also recorded highest multiplication on medium containing 1.5 mg/l Kinetin. The treatment 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivar. Pawar *et al,* 2002 reported effect of growth regulators on in vitro multiplication of sugarcane cultivars (Co-86032, Co-740 and Co-8014) and studied the effect of different levels of kinetin, BAP and coconut water (CW) on shoot multiplication , length of main shoot and number of leaves on main shoot using MS medium supplemented with three levels of kinetin (0.5, 1.0 and 1.5 mg/l), four levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) and two levels of CW (10 and 20%). The

treatment MS + 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivars. Baksha *et al*, 2002, reported multiple shoots from shoot tip explant of sugar-cane (*Saccharum officinarum)* by culturing on MS medium supplemented with BAP (0.5-2.0 mg/l), Kn (0.1-0.5 mg/l). Mamun *et al,* 2004 reported that *in vitro* micro propagation for sugarcane variety viz., Isd-28 and Isd-29 showed best shooting when media were supplemented with BA 1.5 mg /l +0.5 mg /l NAA.

2. b. Rooting

Shenk and Hildebrandt, 1972, have reported requirement of high concentration of auxin for rooting in sugarcane. Barba *et al*, 1977, reported that root development requires higher sugar levels in nutrition media. Nadar and Heinz, 1977, reported that preferred auxin for root initiation was NAA. Rooting was induced in clumps. Chen *et al*, 1988, reported that rooting in sugarcane takes place in the clumps. Auxins are reported to be essential for root induction in sugarcane, 70 gm/l sugar in MS media supplemented with 500 mg/l casein hydrolysate and 5 mg/l of NAA induced rooting. Naritoom *et al,* 1993, initiated rooting on MS medium with 1 mg IBA/l. Dhumale *et al,* 1994, induced roots on half strength MS medium containing 2 mg/l IBA + 1 mg/l IAA. Cheema *et al,* 1995, demonstrated rooting on MS medium supplemented with 7% sugar and 5 mg/l NAA. Singh *et al,* 2001, transferred clumps with 5-10 well grown shoots on 1/2 strength MS liquid medium supplemented with NAA (5.0 mg/l) and elevated sucrose level (60 g/l). All cultures were incubated at 25 ± 2^0C under 12 h illuminations at a photon flux density of 50-70 micron. The development of fine roots, which began after 7-15 days, ranged from 75% (CoJ 85) to 95% (CoJ 86). Profuse rooting was achieved within 30-40 days. Plants transplanted in the field 45-60 days after hardening in the greenhouse showed uniform growth and asynchronous tillering within 60 days after transplanting. Pawar *et al,* 2002, induced rooting (85-92%) by transferring shoot clumps on 1/2 MS medium containing 2 mg/l NAA and 1.0 mg/ IBA. Mamun *et al*, 2004, reported that. Best results of

rooting ware observed on modified MS with auxins. Eighty to ninety percent regenerated plantlets were viable at normal temperature with 85% humidity while transferred sterilized.

Hardening

Pawar *et al,* 2002, reported plantlets kept for 6-7 days in trays for hardening containing coconut peat showed the highest survival percentage (86%) than plantlets hardened on sand, soil rite, sand + soil rite (1:1), or sand + soil rite + soil (1:1:1).

2. c Callus and cell suspension culture

2. c. 1 Callus induction

Sugarcane tissue culture was first initiated in Hawaii in 1961. Nickell, 1964, was the first to report the establishment of callus cultures of sugarcane, which provided the impetus for culturing other genera of Gramineae. Recent studies of the culture of Gramineae species have improved our understanding of the influence of genotype, donor explants and media on the induction and regeneration and regeneration of callus cultures (Bright and Jones, 1985; Ozias-Akins and Lorz, 1984; Vasil, 1983a, b). Most success in prolonging plant regeneration, and, in some cases, the frequency of shoot production from long term cultures, has come from the realization that the Gramineae produce various types of tissues which differ in their totipotency (Nabors, 1983). Thus, maintenance of plant regeneration has been achieved in maize (Lowe *et al*, 1985; Vasil and Lu 1984; Armstrong and Green, 1985), oats (Heyser and Nabors, 1982), rice (Heyser *et al*, 1983) and wheat (He *et al*, 1986), when each subculture was accompanied by visual selection and careful transfer of the correct type of tissue to suitable medium.

Rapid callus formation in sugarcane has been obtained from young leaves, shoot apices and young inflorescences (Heinz and Mee, 1969; Heinz *et al*, 1977; Liu, 1984) but such callus rapidly loses its regeneration capacity when randomly sub cultured and grown in the light (Liu, 1984). However it has been

reported that morphogenic callus was obtained from leaf explants incubated in the dark (Ho and Vasil, 1983a). Barba *et al,* 1977, reported callus formation on medium MS medium supplemented with 3.0-4.0 mg/l 2, 4-D. Chen *et al,* 1988, also reported callus growth using leaf explant from the innermost whorl(s) and cultured on MS media supplemented with 0.5-1.5 mg/l 2, 4-D at 27°C in dark. Modified MS medium supplemented with 2, 4-D for callus induction of sugarcane was reported by Islam *et al,* 1992 and Hossain *et al,* 1996. Cheema *et al,* 1995, reported callus formation in sugarcane variety CoJ-76 on MS media with 3 mg/l 2, 4-D and 0.2 mg/l BAP. Taylor *et al,* 1992, also reported that callus, on leaf explant tissue taken from a range of 18 genetically diverse sugarcane cultivars, was established by culture on MS media containing 13.4 µm 2, 4- D. It was found that the degree of embryogenic callus (EC) depends on the genotype and concentrations of 2, 4-D with some genotypes forming more EC at 2-5 mg/l 2, 4-D than others. Patel *et al,* 2000, reported MS medium supplemented with 3mg/l 2, 4-D and 10% coconut water rich in natural cytokinin gave rise to compact greenish callus within 15-20 days. However, we have observed that greening of calluses only observed upon transfer to regeneration media in light.

2. c. 2 Callus multiplication and regeneration

In most of these studies, callus was induced in the presence of auxin, either 2, 4-D or picloram. To promote regeneration, callus was then transferred to medium with either a reduced or no 2, 4-D (Ahloowalia and Maretzki, 1983). Kaur *et al*, 2001 reported Callus proliferation on the MS medium containing 2, 4-D (4 mg/l) + BAP (0.5 mg/l). Chen *et al,* 1987, reported that sugarcane cultures produced three morphologically distinct types of callus; a white compact callus capable of plant regeneration, a friable non-morphogenic callus and a mucilaginous nodular callus which could revert to these other two types depending on the concentrations of 2, 4-D in the culture medium. Leaf explants formed morphogenic callus more readily than excised stem apices, with most prolific callus formation in the dark. Fitch and Moore, 1990, reported that the proportion of the tissue types depended on the genotype,

amount and kind of auxin. Long-term regeneration of sugarcane callus cultures was maintained by selection of green callus on MS agar medium containing 0.5 mg/l picloram or 2, 4-D. Green callus on picloram media always regenerated green plants.

Histological studies have revealed that plant regeneration from sugarcane tissues occur by organogenesis (Liu and Chen, 1974) or somatic embryogenesis (Ho and Vasil, 1983 a, b). Somatic embryogenesis was reported from young leaves (Ahloowalia and Maretzki, 1983; Ho and Vasil, 1983; Chen *et al*, 1988; Brisibie *et al*, 1997), apical meristem (Ahloowalia and Maretzki, 1983) and immature inflorescences (Liu, 1993; Blanco *et al*, 1997), as well as organogenesis from young leaves (Chen et al, 1988; Fitch and Moore, 1990).

Irwine *et al,* 1991, reported that combining NAA with Kinetin promoted rapid sugarcane regeneration from callus. Taylor *et al,* 1992, found that the degree of embryogenic callus (EC) depended on the genotype and concentrations of 2, 4-D with some genotypes forming more EC at 2-5 mg/l 2, 4-D than others. EC was initiated from all genotypes on MS medium with 3 mg/l 2, 4-D and these calli readily regenerated into plants. Fitch and Moore, 1993, tested media supplemented with different concentration of proline for its effect on embryogenic calluses of sugarcane. The largest quantities of embryogenic calluses were produced on Murashige & Skoog medium, containing 4.5 mM 2,4-dichlorophenoxyacetic acid (2, 4-D). The effect of supplements on somatic embryogenesis was examined. Kinetin (0.5 µM) and 10% (v/v) coconut water in callus initiation medium were inhibitory to subsequent embryogenesis. Embryogenic calli increased in fresh weight on N6 medium supplemented with proline concentration up to 90 mM though maximum number of green plants regenerated at 40 mM proline. Cheema *et al* (1995) reported transfer of plantlets differentiated from calli to MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l Kinetin for shoot proliferation. It was observed by Gupta et al, 1995, that MS salts with 0.5 mg/l NAA + 3.0mg/l Kn + 30g/l sucrose + 0.8% agarose produced 30-50 regenerated plantlets from proto calli. Gupta *et al*, 1995, also reported four distinguishable types of calli. Callus type 1:

organized, yellowish, nodular/globular embryoid containing compact callus; callus type 2: smooth, semi-organized, light yellowish, differentiated callus; callus type 3: spongy-type, soft, whitish, rough surface callus; callus type 4: unorganized, light yellowish, soft, rough surface callus. Callus type 3 and callus type 4 were unable to regenerate the plantlets. However callus type 1 and callus type 2 showed high potential for morphogenesis of sugarcane.

Aftab *et al,* 1996, achieved a good embryogenic callus responses using MS basal medium supplemented with 0.5 mg/l picloram under dark conditions at 27^0 C from young leaves of sugarcane (Saccharum spp. Hybrid cv. Col-54). Islam *et al,* 1996, cultured segments from young leaf bases of 4 sugarcane clones on MS medium supplemented with 10% coconut milk and 0.5-3 mg/l 2, 4-D. Clone isd 16 showed the highest frequency of somatic embryogenesis (78.5%). Upon transfer to regeneration media, the isd 16 showed highest frequency of plant regeneration (87.5%) and produced highest number of plantlets (17.3) per callus. Rodriguez *et al,* 1996, carried out a study on the features of embryogenic and non-embryogenic calli using SEM and found that embryogenic calli are characterized by cells organized into embryoid structures while cells of non-embryogenic calli formed disorganized masses. The effect of exogenous polyamines on somatic embryogenesis had been studied; polyamines such as putrescine, spermine or spermidine, these polyamines controlled phenolics and also increased the induction of embryogenic callus (Sargent *et al*, 1998). Falco *et al*, 1999, reported that plant regeneration occurred only from the nodular and compact calli. Serial sectioning showed that both somatic embryos and adventitious shoots were formed. Anbalagen *et al,* 2000, cultured immature spindle leaves for callus induction on MS media containing 2, 4-D and in combination with kinetin and benzyladenine (BA). The callus tissues derived from 5-month-old leaves were used for regeneration. The regeneration media were based on MS medium with two concentrations of NAA and kinetin. Two types of callus were formed from leaf explants: (a) loose, friable and non-embryogenic (b) compact, white, nodular and embryogenic. Direct regeneration was observed from the leaf explants of all cultivars.

Patel *et al,* 2000, reported MS medium supplemented with 3mg/l 2,4D and 10% coconut water rich in natural cytokinin gave rise to compact greenish callus within 15-20 days. Prajapati and Patel, 2000, had also given a regeneration media for sugarcane variety CoC -671: MS + 0.5 mg/l NAA + 3.0 mg/l Kinetin + 30 g/l sucrose + 0.8% agarose. These regenerants were separated after 15 days and then transferred to MS liquid medium for proliferation of shoot and root development. Most recently rapid shoot regeneration from embryogenic callus was obtained using MS medium containing thidiazuron (Gallo – Meagher and Chengalrayan, 2000). All TDZ treatments resulted in faster shoot regeneration than the Kinetin/NAA treatment and more shoot production than the standard 2, 4-D or Kinetin/NAA treatments. Kaur *et al,* 2001, reported various factors affecting efficient plant regeneration from long term maintained callus culture were investigated in 3 sugarcane varieties e.g. CoJ 64, CoJ 83, CoJ 86. The calluses maintained through subculturing on MS medium supplemented with proline resulted in good somatic embryogenesis. Gill *et al,* 2004, reported that callus formation in sugarcane is explant, genotype and hormone dependent. Supplementation of the control medium independently with maltose (30 mg /l), proline (560 mg/l), abcissic acid (2 mg/l) and reduced auxin i.e., 2, 4-D (3 mg/l) enhanced somatic embryogenesis considerably. Similarly, higher shoot regeneration was achieved on a medium containing only cytokinin, BAP (0.5 mg/l).

2. c. 3 Cell suspension culture

Falco *et al,* 1996, reported plant regeneration was only obtained in young cultures, which were non-homogenous, and in culture media with low 2, 4-D.
Sugarcane cell suspensions were established from young leaf-derived callus in MS medium supplemented with 5% (v/v) coconut water, 3 mg/l 2, 4-dichlorophenoxyacetic acid and 500 mg/l casein hydrolysate. Cell suspension were maintained by partial selection of embryogenic cells , which was done by letting the cell suspension settle briefly , followed by discarding the supernatant and replacing the culture medium up to 35-40 ml every seven

days . Cell suspension growth was measured by embryogenic cell number, packed cell volume and cell fresh and dry matter.

2.d. Sugarcane Improvement

The first report on transgenic sugarcane was in 1992 (Bower and Birch, 1992). Subsequently there have been several reports on sugarcane transformed with marker genes (Bower *et al*, 1996) as well as transgenic sugarcane containing genes controlling such characters as herbicide resistance (Chowdhury and Vasil, 1992; Gallo-Meagher and Irvine, 1996; Enriquez-Obregon *et al*, 1998; Falco *et al*, 2000), insect resistance (Arencibia et al, 1997) and sugarcane mosaic virus resistance (Ingelbrecht *et al*, 1999). Sugarcane mosaic virus resistant plants have also been obtained as somaclonal variants derived from tissue culture (Orpeza *et al*, 1995; Orpeza and Garcia, 1996). Leal *et al,* 1996, reported the somaclonal variation as a source of resistance to eyespot disease of sugarcane.

3. Materials and method

3. a Glassware

The glassware used for culture work comprised of 250 ml, 500 ml and 1000 ml conical flasks, Kasablanka bottles, test tubes, jars, pipettes, measuring cylinder (100 ml, 500ml). The glassware was properly cleaned with teepol detergent, washed under running tap water and left to dry. The glassware was then steam sterilized in an autoclave at pressure of 15 lb/in^2 at 121°C for 20 minutes.

3. b Culture Medium

Media often need to be varied for different plant genera and according to the kind of culture to be undertaken. Many different kinds of plant cultures can be grown on one kind of medium. MS media as reported by Murashige and Skoog (1962) or modified MS media as supplemented with various plant growth regulators have proved to be suitable for callus growth, direct and indirect morphogenesis and shoot culture of sugarcane as reported by several authors.

Stock of major elements (4x conc.), minor elements (1000x conc.) were stored in a refrigerator and were mixed in desired proportions only before use. The basal medium was supplemented with different combination and concentration of the growth regulators such as 6-BAP, alpha-Naphthalene acetic acid (NAA), Indole butyric acid (IBA), 2, 4 dichlorophenoxy acetic acid (2, 4-D) and Indole acetic acid (IAA). Auxins such as 2, 4-D and IAA were dissolved in 1 N KOH and cytokinins were dissolved in 1N HCl before making up the final volume with distilled water. Auxins when added in appropriate concentrations may regulate cell elongation, cell division, formation of adventitious roots, inhibition of adventitious and axillary shoot formation, callus initiation and growth, and induction of embryogenesis.

Cytokinins added in appropriate concentrations may regulate cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation,

inhibit root formation, activate RNA synthesis, and stimulate protein and enzyme activity.

The medium was prepared by adding required amounts of stock solutions and final volume was made up with deionised water. The pH was adjusted in all cases to 5.8 by using 1N KOH or 1N HCl and media were solidified with 0.8% agar (Himedia, India). They were then autoclaved at 121°C for 20 minutes at 15 psi pressure and transferred to the media storage room where they were kept under aseptic conditions till their further use.

IN VITRO CULTURE MEDIA

Basal medium

Murashige and Skoog's (1962) mineral solution (macro and micro-nutrients):

A. Macro-elements (Hi Media)	**Amounts (mg /l)**
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_20$	440
KH_2PO_4	170
$MgSO_4.7H_2O$	370
B. Micro-elements (Hi Media)	
H_3BO_3	6.2
$NaEDTA.2H_2O$	37.30
$MnSO_4.4H_2O$	22.3
$FeSO_4.7H_2O$	27.8
ZnSO4.7H2O	8.6
KI	0.83
$Na_2MoO_2.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

C. Vitamins (Hi Media)

Glycine	2.0
Nicotinic acid	0.5
Pyridoxine	0.5
Thiamine HCL	0.1
Inositol	100

D. SUGAR (Mawana Sugar Ltd.)	3000
E. AGAR	8000

Phytohormones

(Manufacturer/supplier)

Auxins	(sigma)	- IAA, NAA, 2, 4-D
Cytokinins	(sigma)	- 6-BAP, Kinetin
Gibberellins	(sigma)	- GA_3

3.c. Choice of explant

Before micropropagation commences, careful attention should be given to the selection of the stock plant or plants. They must be a typical of the variety or species and free from any symptoms of disease. The kind of explants chosen, its size, age and the manner in which it is cultured can all affect whether tissue culture can be successfully initiated, and whether morphogenesis can be induced. Choice of the correct explant is important when propagation is to be based on direct or indirect shoot initiation. The part of the plant from which explants are obtained depends on:

- The kind of the culture to be initiated
- The purpose of the proposed culture
- The plant species to be used

Explants for culture were obtained from stalk apices of 8-12 months old field grown healthy sugarcane cultivars (CoS 8436 and CoJ 83) maintained at T.I.E.T, Patiala.

3.d Explant sterilization

- Actively growing points of sugarcane top, taken as explants from 8-12 months old sugarcane cultivar.
- The outer mature leaves were removed till a spindle of about 1 cm in dia was obtained. The spindle (3.0-4.0 cm) was excised and thoroughly washed under tap water for 30 minutes.
- The explants were treated with aqueous solution of Bavistin (BASF India Limited) [1% w/v] and few drops of Tween 20 for 20 minutes.
- Explants were again washed with sterile double distilled water for 10 minutes.
- The explants were then taken inside the laminar hood for further sterilization. Inside the laminar hood, sterilization with savlon (Johnson & Johnson) (1.5% v/v chlorohexidine gluconate solution and 3.0% w/v cetrimide) and 0.1% mercuric chloride (Ranbaxy) for 7-8 minutes each is carried out. Finally rinsing is done thrice with sterile double distilled water to completely remove any mercuric chloride.

3.e Initiation and culturing of the spindle

- Sterilized explants were transferred aseptically to the sterile glass plates under the laminar flow hood for making them into suitable sizes (2.0-2.5cm). The outer whorls of the spindle were removed with sterile scalpels without injuring the spindle.
- The spindle was placed in a test tube without touching the rim of the test tube and the lid was replaced onto the test tube and sealed with klinfilm.
- The cultures were incubated in cool white light (GE polylux 2500-3000 lux) for about 16 hours day light and 8 hours night break, temperature of about 25±2°C was maintained in plant growth room at TIFAC CORE.
- After 10-14 days of incubation when the bud break was observed, these cultures in test tubes were aseptically transferred to culture jars.

Different combinations of initiation medium:

MS basal taken as control

S_1M_1 - MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin

S_1M_2 - MS + 1.0 mg/l BAP + 1.5 mg/l Kinetin

3.f Establishment of cultures

Explants were serially transferred into semi solid medium once bud break(s) were observed. The original spindle portion is gradually removed and new buds sprout from the base turning into a small mass of plantlets in two cycles. After two cycles of transfer of the spindles, the spindles had established themselves. The initiated plants were taken out of the test tube, medium adhered to the plants was removed, undesirable / brownish leaves were removed and these plants were then transferred to the culture bottles containing autoclaved semi solid medium 50 ml in each bottle. Seal the cultures and incubate them in the culture room under the standard conditions of temperature (25±2°C) for 16 hours (2500-3000 lux) and humidity (approx 85%)

3.g Multiplication by axillary shoots proliferation

Micropropagation through axillary shoot proliferation is the most reliable technique for mass propagation since it ensures genetic stability of the clones. The axillary buds are cultured in medium supplemented with high cytokinin concentration to break bud dormancy and produce axillary shoots which can be subdivided into smaller clumps of shoots which in turn develop similar clusters after subculture on fresh medium.

Multiplication protocol

- Several shoots/ shoot clumps were transferred from the culture bottle to a sterile glass plate using sterilized scalpel. Brownish coloured leaves of the clump were pruned and the clump was sub divided into the smaller clumps.

- Mass of the plantlets was transferred into a new culture flask containing different combinations of liquid multiplication media (30 ml). All this work was done with extreme care inside the laminar hood to avoid any possible chance of contamination.
- Sealed the cultures and incubated in the culture room under light and repeated the multiplication cycle 2-3 times after every 14 days so that numbers of shoots were obtained from each explant increase substantially.
- Observed the culture at the end of each culture passage and recorded the number of shoots and average height of the shoots obtained from each explant piece.

Different combinations of multiplication medium:
MS basal media taken as control
SM_1 - MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin
SM_2 - MS + 0.2 mg/l BAP + 1.0 mg/l Kinetin
SM_3 - MS + 1.0 mg/l BAP + 1.5 mg/l Kinetin

3. h Rooting of plantlets

Once the sufficient numbers of shoots have been generated, steps are taken to grow individual plantlets capable of carrying out photosynthesis and survival without any artificial supply of carbohydrates. It was found that in sugarcane, rooting is promoted in high concentration of sugar and auxin, it is necessary to transfer shoot clumps or separated shoots to medium containing higher salts and sugar, resulting in the root formation.

Rooting of shoots

- In the laminar flow, under sterile conditions, the parafilm and cap were removed from the culture bottle and with the help of forceps, carefully removed the explant from the multiplication medium.
- Placed the multiplied shoot mass on sterile glass plate. Using a sterile scalpel carefully removed or divide small clumps of shoots. Undesirable

portion of the explant were removed and using sterile forceps rinsed in 70% ethanol and flamed, shoot clumps were carefully placed into the liquid rooting medium.

- Proper care was taken that the lower side of the shoot remain in contact with the media and shoots remain straight. Cultures were sealed and incubated for 2-4 weeks until roots appear. Once rooting is completed, observations were recorded for average height of 5 shoots/clump, no. of rooted shoots and root length.

Different combinations of the medium for rooting:
MS basal media (liquid) as control
SRM_1 - MS + 3 mg/l NAA+ 30 gm/l sugar
SRM_2 - MS + 5 mg/l NAA +70 gm/l sugar

3. I Transplantation and hardening of plantlets

In vitro grown plants under controlled conditions produces delicate shoots and plantlets. These plantlets suffer high mortality if they are planted directly from the culture vessel into the natural environment. The transition of plantlets from completely controlled conditions should be gradual in order to prepare plants to survive in the field condition, this is called acclimatization. The delicate nature of plant material raised in vitro arises because, when cultured in conditions of comparatively low light intensity and high relative humidity, the anatomy and physiology of tissues are different to those of plants grown in the green house or field. The plants, although green in colour do not prepare sufficient food for their survival, also the natural protective covering of cuticle is not fully developed due to high humidity inside the culture vessel. The roots of the plants are gently washed to remove the agar sticking to them. The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (approx 90%) and it is gradually reduced over a period of 6-7 weeks. During this time the plant attain ability to synthesize more food and develop cuticular covering. Plants are then maintained under shade and are ready to be used in open nursery.

Protocol for transplantation and hardening

- Gently remove well-rooted plantlets from the culture vessel keeping the roots intact by using forceps with extreme care to avoid any mechanical damage to the plantlets.
- Plantlets were thoroughly washed with in tap water to remove any remaining medium possibly on them. Further plantlets were given Bavistin treatment (1gm/l) for 10 minutes to protect the plant from any near future fungal attack.
- Planted the regenerants in hykotray containing soil and agropeat in 1:4 (v/v). Plants were thoroughly watered and kept in poly house under humidity range of 75-85% and temp of 26-28 ^{0} C for about 2-3 week. These plantlets were then transferred to shade house (75%) in which they would be kept under the humidity range of 60-70% with intermittent sprinkling of water for approx 2 weeks.
- The plants are then transferred to open nursery for final stage of acclimatization for another 2 weeks. When the plants have become well acclimatized they are given fertilizer treatment. Success of hardening protocol was determined by calculating survival %. Plants are ready for field transplantation after 6-7 weeks.

3. j Callus culture and multiplication

Callus is an actively dividing non-organized mass of undifferentiated and differentiated cell tissue that develops on or around an wounded or cut plant surface or that develops during tissue culture of plant parts. Callus formation from explant tissue involves the development of progressively more random planes of cell division, less frequent specialization of cells and loss of organized structures (Thorpe 1980; Wagley *et al* 1987). The technique of callus culture was first developed in the late 1920's and 1930's and was one of the primary methods of tissue culture for many years. Callus can be initiated in vitro by placing small pieces of the whole plant (explants) onto a growth supporting medium under sterile conditions. Under the stimulus of

endogenous growth substances or growth regulating chemicals added to the medium the metabolism of cells, which were in a quiescent state, is changed and they begin active division. During this time differentiation and specialization, which may have been occurring in the intact plant, are reversed and explant gives rise to new tissue, which is composed of meristematic and unspecialized cell types.

Callus induced from explants differ considerably in morphology: spherical, semi-spherical, plate like or amorphous. Their consistency may be compact, hard or loose, friable or dissociated. Based on the appearance of calli, they are classified as compact calli and friable calli. The individual cells of each parenchymatous mass are having the ability to form a whole plant. This potentiality of generation of an entire plant from a single cell is known as Totipotency. Based on this ability, the calli are described as calli having regeneration potential and calli without regeneration potential, in other words as embryogenic calli and non-embryogenic calli respectively. The embryogenic calli have a smooth, white knobby appearance and are composed of small isodiametric cells, while non-embryogenic calli are yellow or translucent, wet, rough and are composed of larger elongated cells. Callus induced from the explants can be isolated and maintained for any number of years by transferring the callus pieces to fresh medium supplemented with balanced auxin cytokinin ratio and other additives. This step is termed as Subculture. Properties and consistency of callus tissues change easily during each subculture. Very frequently the tissue dies after three or four passage due to the exhaustion of some vital metabolites. However, the aging of callus affects proliferation after prolonged subculture.

Explant sterilization

- Actively growing points of sugarcane top, taken as explants from 6-12 months old sugarcane cultivar.
- The outer mature leaves were removed till a spindle of about 1 cm in diameter was obtained.

- The basal part of the spindle was excised and thoroughly washed with tap water for 30 minutes.
- The explants were treated with fungicide (Bavistin) and soap solution (Tween 20) for 20 minutes.
- Explants were again washed under running tap water for 10 minutes.
- The explants were then taken inside the laminar hood for further sterilization. Inside the laminar hood, sterilization with savlon and 0.1% $HgCl_2$ for 7-8 minutes each is carried out. Finally rinsing is done thrice with double distilled water to completely remove any mercuric chloride.

Callus induction

- Sterilized explants were transferred aseptically to the sterile glass plate. The outer 2 or 3 whorls of leaves were removed using a sharp scalpel blade.
- Innermost leaf whorls were cut to 2.0-2.5 cm and the cut piece injured obliquely at 2-3 places was used as an explant for culturing.
- Leaf sections were placed with basal cut ends in contact with culture media.

  Cultures were maintained at 25±2°C in the dark.

Media for callus induction:

MS basal media taken as control

CIM $_1$ - MS + 1 mg/l 2, 4-D

CIM $_2$ - MS + 2 mg/l 2, 4-D

CIM $_3$ - MS + 3 mg/l 2, 4-D

CIM $_4$ - MS + 4 mg/l 2, 4-D

Callus multiplication

Callus was subcultured regularly after 21 days using visual observations to select morphogenic tissues prior to transfer.

Callus multiplication medium:

CMM $_1$ - MS + 0.5 mg/l 2, 4-D

CMM $_2$ - MS + 1.0 mg/l 2, 4-D

CMM $_3$ - MS + 1.5 mg/l 2, 4-D

CMM $_4$ - MS + 2.0 mg/l 2, 4-D

Growth determination

Established callus cultures were multiplied for two cycles and then callus of 1 cm^2 was inoculated into 4 different media to estimate the growth pattern of callus. The initial fresh weight of three replicates each for 4 treatments of callus was determined. The callus pieces were dried in an oven at 60°C for 2 hours and dry weight was determined. Rests of the cultures were incubated at 25°±2°C in dark conditions. Fresh and dry weights were determined after 7, 14, 21, 28 days and growth curve was plotted.

3. k Regeneration from callus

Callus was segregated on the basis of embryogenic callus and non-embryogenic callus. Only creamish white, nodular embryogenic callus that has regeneration potential was put on different regeneration media. The cultures were incubated in light at 25°±2°C under light (2500 lux).

Media for callus regeneration

RGM_0 - MS basal media were taken as control

RGM_1 - MS + 0.5 mg/l BAP

RGM_2 - MS + 0.5 mg/l BAP + 1.0 mg/l NAA

RGM_3 - MS + 0.5 mg/l BAP + 0.125 mg/l Kinetin + 0.5 mg/l 2, 4-D

RGM_4 - MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin

4. Results and Discussion

4. a Micropropagation

Surface sterilization and aseptic culture establishment-

The primary establishment of the aseptic cultures *in vitro* needed surface sterilization of the explants from *ex vitro* or field grown plants. The outer mature leaves were removed till a spindle of about 1-cm in diameter was obtained. The spindle (3.0-4.0 cm) was then excised and thoroughly washed under running tap water for 30 minutes. The explants were treated with aqueous solution of Bavistin (BASF India Limited) [1% w/v] and few drops of Tween 20 for 20 minutes. Explants were again washed with sterile double distilled water for 10 minutes. They were then taken inside the laminar hood for further sterilization. Inside the laminar hood, sterilization with savlon (Johnson & Johnson) and 0.1% mercuric chloride (Ranbaxy) for 7-8 minutes each is carried out. Finally rinsing is done thrice with double distilled water to completely remove any mercuric chloride.

Mamun et al (2004) reported that sterilization of leaf explant with 0.1% mercuric chloride for 8 to 10 min gave satisfactory results with, 85-90% of the explants being contamination free. However we found that spindles turned black when leaf explants were sterilized with 0.1% mercuric chloride for 8 to 10 min. Chen *et al*, 1998, has described surface sterilization of leaf explants with 95% ethanol for 5 minutes. Chengalrayan and Gallo-Meagher, 2001, reported surface sterilization of leaf explant by 0.5% sodium hypochlorite for 20 minutes.

The sterilization procedure adopted for the sugarcane spindle sterilization is found to be reliable. The aseptic culture initiation % 81.25% for CoJ 83 and was 78.15% for CoS 8436. After the incubation of the spindles in the controlled conditions of temperature (25±2°C), humidity and light intensity (2000-2500 lux, for 16 hours), spindles had turned green from pale yellow.

The experiment was performed 4 times with 5 replicates per treatment.

Table 1: Observation of aseptic culture percentage

Variety	Aseptic culture percentage
CoS 8436	78.15%
CoJ 83	81.25%

For two cycles, spindles were subcultured in the solid media for culture establishment. The swelling was observed after 5 days in CoS 8436 and 8 days in CoJ 83. The bud break of shoots was observed on the spindle after 7 days in CoS 8436 and in 10 days in CoJ 83. Sprouting was observed the base of the spindle. The Browning of the culture media was observed near/around the base of the plant due to the release of phenolics. To avoid this, spindles were subcultured regularly at an interval of 12-14 days by transferring to the fresh medium; base of the spindle was cut and cleaned. Gradually the spindle portion was cut with each subculture and was non existent after 3rd subculture stage. An increase in the size of the bud (1-1.5 cm) was observed after 14 days with a change of colour from yellow to green. A single flag leaf was observed after 21 days with an average height of 2.5-3.0 cm. An average of 4-5 shoots with an average height of 3.5-4 cm was formed after 28 days. Multiple shoot formation occurred after 25-28 days. Three initiation media; S_1M_1 (0.5 mg/l BAP + 0.5 mg/l Kn) and S_1M_2 (1.0 mg/l BAP + 1.5 mg/l Kn) along with MS basal were taken as control. The experiment was performed 4 times with 5 replicates per treatment, initial bud breakage %age, average shoot length and height of shoots was observed. Observations were taken after 14 days before subculturing.

Table 2: Observation of initiation on different media combinations

Medium	Contamination		Bud breakage (14 days)		No. of shoots/ jar (28 days)		Avg. height of shoots (28days)	
	CoS 8436	CoJ 83	CoS 8436	CoJ 83	CoS 8436	CoJ 83	CoS 8436	CoJ 83
MS	0.25 ± 0.50	0.22 ± 0.32	0.35 ± 0.02	0.33 ± 0.22	1.25 ± 0.70	1.05 ± 0.56	2.80 ± 0.42	2.40 ± 0.60
S_1M_1	0.16 ± 0.03	0.23 ± 0.06	0.95 ± 0.10	0.80 ± 0 .07	3.75 ± 0.60	3.50 ± 0.50	4.0 ± 0.54	3.80 ± 0.11
S_1M_2	0.30 ± 0.15	0.26 ± 0.05	0.80 ± 0.21	0.65 ± 0.23	5.75 ± 0.12	4.25 ± 0.34	3.50 ± 0.90	3.20 ± 0.27

In MS basal (control), %age of bud breakage is not significant, as only 35% explants in CoS 8436 and 33% in CoJ 83 show bud breakage within 14 days. It was observed that S_1M_1 (MS + 0.5 mg/l BAP + 0.5 mg/l Kn) was found to be the better initiation medium in terms of bud breakage, average height, and lower contamination %age. A bud breakage of 95% in CoS 8436 and 80% in CoJ 83 was observed. However, average number of shoots, 3.75 in CoS 8436 and 3.5 in CoJ 83 was observed in this media in the initiation stage. Bud breakage % was less in S_1M_2 (MS + 1.0 mg/l BAP + 1.5 mg/l Kn). It was 80% for CoS 8436 and 65% in CoJ 83. However it showed better average no of shoots/explant, 4.0±0.54 with a shoot length (cm) of 3.8±0.11 for CoS 8436 and av. shoots/explant 3.5±0.9 with a shoot length of 3.2±0.27 for CoJ 83 within 28 days. It was observed that higher shoot length as induced on each explant is associated with lesser no of initiated shoots/explant. It was also observed that S_1M_2 with higher cytokinin conc., show better shoot proliferation during axillary shoot initiation.

4.b Shoot multiplication results:

After two cycles of subculturing in the solid media, spindles were transferred to combinations of liquid multiplication media and compared the multiplication rates. Multiplication of shoot cultures was carried out by culturing mother

clusters developed *in vitro*. The effect of the presence of two cytokinins, BAP and Kinetin in MS medium was examined during the phase of propagation. Baksha *et al*, 2002, reported Multiple shoots from shoot tip explant of sugarcane (*Saccharum officinarum)* by culturing on MS medium supplemented with BAP (0.5-2.0 mg/l), Kn (0.1-0.5 mg/l). Three mediums, SM_1 (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin), SM_2 (MS + 0.2 mg/l BAP + 1.0 mg/l Kinetin) and SM_3 (MS+ 1.0 mg/l BAP + 1.5 mg/l Kinetin) with MS basal as control were used for shoot multiplication. Final observation was taken at 3rd subculture stage in multiplication. Observations were taken for evaluating the growth of shoots by taking parameters like average shoot length and number of shoots at 7, 14, 21 days after inoculation. Axillary shoots initiated from the base; progressively increases in size in each subculture and number of shoots multiplied vary from medium to medium.

The experiment was designed as the following, 4 different shoot multiplication medium were chosen, each with 3 culture jar. Each culture jar was inoculated with 2 clumps (4-6 shoots/clump, shoot size appox 3.5-4.0cm). The experiment was conducted four times.

Table 3: Observation of different media combination for multiplication (CoS 8436)

Media	No. of shoots/jar 7 days	Avg. height (cm) of shoots 7 days	No. of shoots/jar 14 days	Avg. height (cm) of shoots 14 days	No. of shoots/jar 21 day	Avg. height (cm) of shoots 21 day
MS	5.20 ± 0.19	4.0 ± 0.57	7.0 ± 0.42	5.5 ± 0.02	12 ± 0.28	6.8 ± 0.58
SM_1	5.80 ± 0.11	4.5 ± 0.22	9.0 ± 0.70	6.9 ± 0.14	15 ± 0.25	9.6 ± 0.02
SM_2	6.12 ± 0.44	4.6 ± 0.09	10.0 ± 0.42	6.0 ± 0.08	19 ± 0.36	8.8± 0.08
SM_3	6.00 ± 0.15	4.2 ± 0.09	12.0 ± 0.31	5.8 ± 0.09	24 ± 0.22	7.4± 0.06

In CoS 8436, an average of 12±0.28 shoots in MS, 15± 0.25 in SM_1, 19± 0.36 in SM_2 and 24± 0.22 in SM_3 were obtained after 21 days. The average height (measured in cm) of the shoots after 21 days was found to be 6.8±0.58 in MS, 9.6± 0.02 in SM_1, 8.8± 0.08 in SM_2, 7.4± 0.06 in SM_3.

In CoJ 83, an average of 10±0.30 shoots in MS, 14±0.25 in SM1, 17± 0.36 in SM_2 and 20± 0.22 in SM_3 were obtained after 21 days. The average height of the shoots (cm) after 21 days was found to be 6.2±0.27 in MS, 8.2±0.02 in SM_1, 7.7±0.08 in SM_2, 7.0±0.06 in SM_3.

Table4: Observations of different media combinations for multiplication (CoJ 83)

Media	No. of shoots/jar 7 days	Avg. Height of shoots 7 days (cm)	No. of shoots/jar at 14 day	Avg. height of shoots (cm)	No. of shoots/ jar at 21 day	Avg. Height of shoots 21 day (cm)
MS	5.0 ± 0.07	3.9 ± 0.90	7 ± 0.28	5.1 ± 0.25	10 ± 0.32	6.2 ± 0.27
SM_1	5.6 ± 0.05	4.2 ± 0.35	9 ± 0.27	6.1 ± 0.11	14 ± 0.19	8.2 ± 0.50
SM_2	5.8 ± 0.11	3.9 ± 0.05	9 ± 0.56	5.8 ± 0.75	17 ± 0.58	7.7 ± 0.05
SM_3	5.5 ± 0.50	4.0 ± 0.48	12 ± 0.89	5.4 ± 0.24	20 ± 0.15	7.0 ± 0.27

A difference in the multiplication rates and no. of shoots obtained at the end of multiplication subculture (21 days) were observed between different varieties. The variety CoS 8436 was found to be more responsive for multiplication. In CoS 8436 the no. of shoots have attained a multiplication rate of 2.3(MS), 2.58 (SM1), 3.10(SM2), 4(SM3) whereas multiplication rate of CoJ 83 was 2.0 (MS), 2.5 (SM_1), 2.93 (SM_2), 3.63 (SM_3) after 21 days. Similarly the rate of increase in shoot height in CoS 8436 was 1.7 (MS), 2.13 (SM_1), 1.9 (SM_2), 1.76 (SM_3) and in CoJ 83 was 1.58 (MS), 1.95 (SM_1), 1.97 (SM_2), 1.75 (SM_3). In control experiments, shoot proliferation rate was significantly lower than the other mediums containing different concentrations of cytokinin indicating significant role of cytokinin in shoot multiplication. It was found that shoot height is higher in SM_1 (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin) than SM_2 (MS + 0.2 mg/l BAP + 1.5 mg/l Kinetin) and SM_3 (MS+ 1.0 mg/l BAP + 1.5 mg/l Kinetin) though shoot proliferation rate was comparatively lower than the other two, indicating that at lower concentration of BAP and Kinetin there was decrease in the multiplication rate. This result was also supported by Chen *et al,* 1987, that at lower concentration of BAP (0.5 mg/l) and Kinetin (0.5 mg/l)

there was loss of proliferation and vigour. SM3 having high concentration of both BAP and Kinetin showed higher shoot proliferation rate but average shoot length was less. Thus with progressive increase in Kinetin concentration there was steady decrease in shoot length. It was also observed that with increase in the height of the shoots there was substantial decrease in the no. of shoots/clump and consequently the multiplication ration. Ali and Afghan, 2001, also reported that basal medium (MS) supplemented with BAP and kinetin gave rapid shoot multiplication. Patel *et al*, 2001, also recorded highest multiplication on medium containing 1.5 mg/l Kinetin. The treatment 1.5 mg/l kinetin + 1.0 mg/l BAP + 20% CW gave the highest values for length of main shoot in all cultivars. However, for convenience, we have not used Coconut water, still we observed approx 3.7(CoJ 83)-4.0 (CoS 8436) fold increase in shoot multiplication rate.

4.c Rooting results:

Once the cluster size and shoot length reaches optimum level, shoot clumps were put into one cycle with half strength MS liquid media to reduce cytokinin carry over effect for 10 days as rooting occur less frequently in high (endo/exogenous) concentration of cytokinin. Nadar and Heinz, 1977, reported that preferred auxin for root initiation was NAA. Root initiation was noted after 5 days and within 12 days profuse rooting was observed. Rooting was induced in clumps. Chen *et al,* 1988, reported that rooting in sugarcane takes place in the clumps. Pawar *et al*, 2002, induced rooting (85-92%) by transferring shoot clumps on 1/2 MS medium containing 2 mg/l NAA and 1.0 mg/l IBA. Mamun *et al,* 2004, reported that better results of rooting were observed on modified MS with auxins. Eighty to ninety percent regenerated plantlets were viable at normal temperature with 85% humidity.

Small shoot clumps of the *in vitro* grown proliferating culture were cultured on 2 rooting medium; SRM_1 (MS+ 3mg/l NAA + Sugar 30 gm/l) and SRM_2 (MS + 5mg/l NAA + Sugar 70 gm/l). Observations were taken after 2 weeks of shoot length, root length and no of roots/clump. The experiment was designed as

the following, 3 different root multiplication medium were chosen, each with 3 culture jar. Each culture jar was inoculated with 2 clumps (4-6 shoots/clump) and observation taken after 14 days.

Table 5: Observations of rooting in different media

Media	Avg. ht (cm) of 5 shoots/clump at 14 days		No. of rooted shoots /jar		Avg. root length (cm)	
	CoS 8436	**CoJ 83**	**CoS 8436**	**CoJ 83**	**CoS 8436**	**CoJ 83**
MS	6.8 ± 0.02	5.9 ± 0.40	-	-	-	-
SRM_1	8.4 ± 0.50	6.6 ± 0.15	8 ± 1.00	7 ± 0.75	1.2 ± 0.14	0.9 ± 0.34
SRM_2	9.2 ± 0.31	7.7 ± 0.19	12 ± 1.99	10 ± 2.00	2.0 ± 0.09	1.4 ± 0.04

No rooting was observed in MS media. Barba *et al*, 1977, also reported that root development requires higher sugar levels in nutrition media. The high concentration of auxin and sucrose showed significant effect on root development as maximum number of roots/shoot clump/jar and root length was observed in SRM_2 medium. In CoS 8436, an average of 12±1.99 shoots were obtained with an average root length (cm) of 2.0±0.09 and in CoJ 83 an average of 10±2.0 roots/shoots were obtained with an average root length 1.4±0.04 (cm). Root developed in SRM_1 medium containing lower concentration of NAA and sugar was poor in quality. The number of shoots rooted and average height of shoots in clump was higher in CoS 8436. In CoS 8436, an average of 8± 1.00 roots/clumps were obtained with an average root length (cm) of 1.2±0.14 and in CoJ 83 an average of 7±0.75 shoots/clumps were obtained with an average root length 0.9± 0.34 (cm) of shoots rooted in clumps. Shenk and Hildebrandt, 1972, have also reported requirement of high concentration of auxin for rooting in sugarcane. Cheema *et al,* 1995, demonstrated rooting on MS medium supplemented with 7% sugar and 5 mg/l NAA. Singh *et al,* 2001, transferred clumps with 5-10 well grown shoots on 1/2 strength MS liquid medium supplemented with 5.0 mg/l NAA and elevated sucrose level (60 g/l). These reports also substantiated our observation that microshoots in clumps of in vitro grown sugarcane plants

require high salt media (higher NAA >3 mg/l) and sugar supplementation (best rooting observed at 70gm/l).

4.d Transplantation and hardening

After 14 days of culture on MS medium meant for rooting, the sufficiently rooted plantlets were transplanted to small hykotrays for hardening prior to their final transfer to the soil. The rooted clumps/plantlets were gently removed from the culture vessel keeping the roots intact by using forceps with extreme care to avoid any mechanical damage and further the plantlets were separated from the shoot clumps. Plantlets were thoroughly washed with in tap water to remove any remaining medium possibly on them. Further plantlets were given Bavistin treatment (1gm/l) for 10 minutes to protect the plant from any near future fungal attack. Planted the regenerants in hykotray containing soil and agropeat in 1:4 (v/v). Plants were thoroughly watered and kept in poly house under humidity range of 75-85% and temp of 26-28 ^{0} C for about 2-3 week. These plantlets were then transferred to shade house (75%) in which they would be kept under the humidity range of 60-70% with intermittent sprinkling of water for appox 2 weeks. The plants are then transferred to open nursery for final stage of acclimatization for another 2 weeks. When the plants have become well acclimatized they are given fertilizer treatment. Success of hardening protocol was determined by calculating survival %. Plants are ready for field transplantation after 6-7 weeks. Plants are the nursery stage and the hardening process could not be completed during the time of preparation of the thesis.

4.e Callus induction

Callus cultures were established by initiating spindle leaf on MS medium supplemented with different concentration of auxin (2, 4-D) taking MS basal as the control. Four different media combinations were used CIM_1 (MS + 1 mg/l 2, 4-D), CIM $_2$ (MS + 2 mg/l 2, 4-D), CIM $_3$ (MS + 3 mg/l 2, 4-D), CIM $_4$ (MS + 4 mg/l 2, 4-D). The outer 2-3 whorls of the leaves were removed.

Innermost leaf whorls of 2.0-2.5 cm in size were injured obliquely at 2-3 places randomly and then inoculated into the media. Culture bottles were incubated in dark at 25±2ºC.

Unwhorling of the leaves, along with swelling of the explant was observed after 2-3 days of culturing. The explant turned pale in colour. Callus initiation starts from the cut edges or injured sites and gradually callus growth occurs completely over the explant. Ho and Vasil, 1983a, also reported that morphogenic callus was obtained from leaf explants incubatedin the dark.

Some of the explants in culture bottles turned green in colour (probably some exposure to light) and did not showed any swelling and callus induction. CoS 8436 exhibited early callus initiation within 8-10 days, whereas CoJ 83 took 12-14 days. Fully-grown callus formation on the leaf spindle explant was observed within 25-28 days. A non-compact, mucilaginous whitish callus was first to develop. Subsequently a compact creamish white, morphogenic callus developed over the entire explant. The 2nd and 3rd innermost whorl showed more callus induction than 1st, 4th and 5th whorl. Chen *et al,* 1988, also reported callus growth using leaf explant from the innermost whorl(s) and cultured on MS media supplemented with 0.5-1.5 mg/l 2, 4-D at 27°C in dark. The experiment was designed as the following, 5 different callus induction medium were chosen, each with 3 culture jar (as replication). Each culture jar was inoculated with 3 explants (leaf spindles). The experiment was conducted four times. Observation was taken after 28 days.

Table 6: Observations on callus induction.

Media	% of Explants showing callus induction in		Degree of callus	Texture of callus
	CoS 8436	CoJ 83		
MS	No Induction	No induction	-	-
CIM $_1$	44	42	+	Non com, mu, w
CIM $_2$	67	62	++	Com, nod, cr. w
CIM $_3$	85	78	+++	Com, nod, cr. w
CIM $_4$	72	66	+++	Com, nod, cr. w

(Non com: Non compact; Com: Compact; Nod: Nodular; Mu: mucilaginous; Cr w: creamish white; w: white)

Callus formation occurred on all media except MS basal taken as control. However, the explants on MS medium showed slight swelling but subsequently they turned brown resulting in death of the explant. Thus it observed that presence of auxin (2, 4-D) is essential for callus induction. Modified MS medium supplemented with 2, 4-D for callus induction of sugarcane was also reported by Islam *et al,* 1992 and Hossain *et al,* 1996. The percentage of callus induction and appearance of callus varied among different medium in both the varieties. CIM_1 showed poor response towards callus induction as only 44% of explants of CoS 8436 and 42% of explants of CoJ 83 showed callus formation after 28 days. Also the callus formed on CIM_1 was non compact, mucilaginous and watery, whereas compact, nodular creamish white callus was formed in all other 3 medium. In CIM_2, 67% explant of CoS 8436 and 62% of CoJ 83 showed callus formation. However, culture media CIM_3 and CIM_4 enriched with 2, 4-D at 3 and 4 mg/l showed comparatively better response towards callus induction. Callus induction % was highest in CIM_3 with 85% induction in CoS 8436 and 78% in CoJ 83 followed by CIM_4 which showed 72% in CoS 8436 and 66% in CoJ 83. This shows that callus induction gradually enhanced with increase concentration of 2, 4-D. This observation was supported by Barba *et al,* 1977, as callus formation was observed when supplemented with MS medium with 3.0-4.0 mg/l 2, 4-D. However, there is slightly a difference in callus induction/formation genotypically. Taylor *et al,* 1992, also reported d that the degree of embryogenic callus (EC) depended on the genotype and concentrations of 2, 4-D with some genotypes forming more EC at 2-5 mg/l 2, 4-D than others. Patel *et al,* 2000, reported MS medium supplemented with 3mg/l 2, 4-D and 10% coconut water rich in natural cytokinin gave rise to compact greenish callus within 15-20 days. However, we have observed that greening of calluses only observed upon transfer in light.

In the process of callusing the callus tissue excreted phenols into the medium, as a result, media turned black/brownish; growth of the calli slowed down and calli could not survive if grown on the same medium for more then 6 weeks. To avoid this calli was transferred to the fresh medium at regular intervals.

4.f Callus multiplication

It has been reported that though high concentration of 2, 4-D is required for callus induction but multiplication of callus is carried out at reduced or no 2, 4-D (Ahloowalia and Maretzki, 1983). It is assumed that elevated levels of auxin hindered the development of pro-embryogenic callus into embryogenic callus and the pro embryogenic callus was transformed into non-embryogenic callus. After 4-5 weeks of incubation in callus induction media, primary calli obtained were excised and transferred to the half concentrations of each respective media. Chen *et al,* 1987, also reported that low 2, 4-D concentrations in the culture medium were essential in maintaining the morphogenic callus. 4 different media compositions were taken CMM_1 (MS + 0.5 mg/l 2, 4-D), CMM_2 (MS + 1.0 mg/l 2, 4-D), CMM_3 (MS + 1.5 mg/l 2, 4-D), CMM_4 (MS + 2.0 mg/l 2, 4-D).

Only the calli that were considered embryogenic were transferred to fresh media. Embryogenic callus was characterized by physical appearance. The creamish white, compact, nodular calli were considered embryogenic, where as mucilaginous, watery and whitish calli were considered to be non-embryogenic. This was done as Chen *et al,* 1987, (and also supported by Gosal *et al*, 2004) reported that sugarcane cultures produced three morphologically distinct types of callus, a white compact callus capable of plant regeneration, a friable non-morphogenic callus and a mucilaginous, nodular callus which could revert to these other two types depending on the concentrations of 2, 4-D in the culture medium. Leaf explants formed morphogenic callus more readily than excised stem apices, with most prolific callus formation in the dark.

In both the varieties, a considerable mass of callus was accumulated within 4-5 weeks. Established callus cultures were then multiplied for 2 cycles by serial subculturing in multiplication media. Subculturing was done regularly at an interval of 21 days. Callus of 1 cm^2 area was then inoculated into the fresh media and cultures were used to estimate the growth pattern of the callus. The initial fresh weight of three replicates each for 4 treatments of callus was

determined. The callus pieces were then dried in oven at 60ºC for 2 hrs and dry weight was determined (Rests of the cultures were further incubated at 25°C in dark conditions. Fresh and dry weights were determined after 7, 14, 21, 28 days and growth curve was plotted (as described by Falco *et al*, 1996). The experiment was done once due to lack of time we can't repeat these experiments for further confirmation of these results.

Table 7: Observations of callus multiplication (CoS 8436).

(Avg. fresh weight in grams)

Media	Fresh wt. (gm) (7 days)	Fresh wt. (gm) (14 days)	Fresh wt. (gm) (21 days)	Fresh wt. (gm) (28 days)
CMM_1	0.97 ± 0.09	1.32 ± 0.18	1.60 ± 0.06	1.56 ± 0.06
CMM_2	1.06 ± 0.02	1.46 ± 0.20	1.73 ± 0.02	1.70 ± 0.02
CMM_3	1.38 ± 0.14	1.76 ± 0.12	2.01 ± 0.10	1.98 ± 0.10
CMM_4	1.26 ± 0.30	1.61 ± 0.14	1.83 ± 0.04	1.79 ± 0.04

Table 8: Observations of callus multiplication (CoS 8436).

(Avg. dry weight in grams)

Media	Dry wt. (gm) (7 days)	Dry wt. (gm) (14 days)	Dry wt. (gm) (21 days)	Dry wt. (gm) (21 days)
CMM_1	0.07 ± 0.02	0.11 ± 0.08	0.13 ± 0.04	0.12 ± 0.04
CMM_2	0.08 ± 0.03	0.12 ± 0.11	0.14 ± 0.02	0.14 ± 0.02
CMM_3	0.10 ± 0.05	0.14 ± 0.02	0.18 ± 0.11	0.17 ± 0.11
CMM_4	0.09 ± 0.06	0.14 ± 0.03	0.16 ± 0.06	0.15 ± 0.06

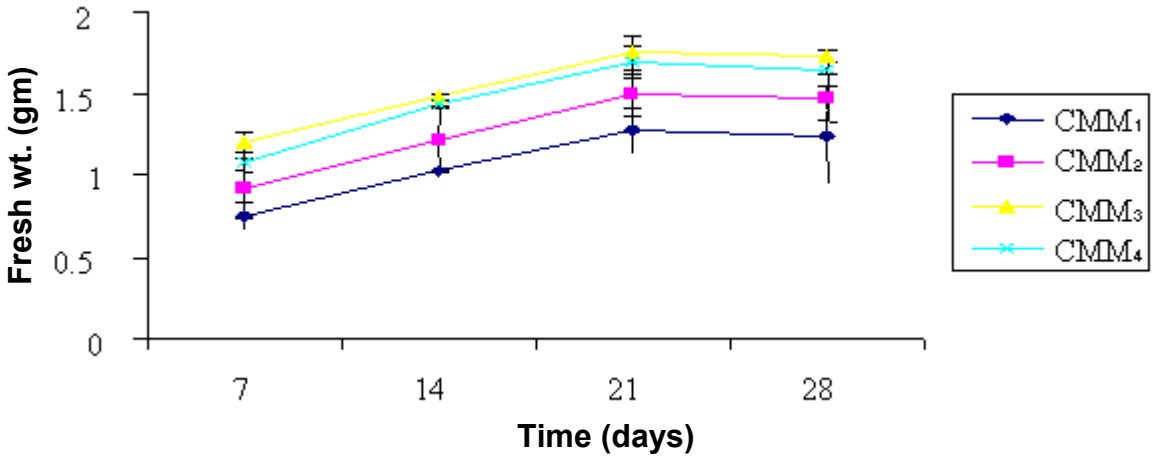


Figure: 5 Callus growth curve of (CoS 8436) on different media used

It was observed that all the medium combination showed a consistent increase upto 21 days then it somewhat decreased for fresh weight comparisons. Also the proliferation of callus was highest in 2.01 ± 0.1gm (CMS_3) for CoS 8436 and1.75 ± 0.12 gm (CMS_3) for CoJ 83 followed by 1.83 ± 0.04 (CMS_4) for CoS 8436 and1.69 ± 0.14 gm (CMS_4) for CoJ 83. Thus indicating that the callus multiplication (fresh wt/dry wt) was better in CMS_3 than CMS_4 and other multiplication/maintenance medium. Eventually there was decrease in the rate of callus multiplication and it was observed that mass of compact nodular callus turning brown in color and will never revert to original appearances even after subculturing. It is also observed that even careful selection and inoculation of compact/nodular calluses, some sectors of mucilaginous, white callus appeared during proliferation. These were discarded every time on subculturing. Thus to maintain the embryogenecity, callus was transferred to fresh medium at a regular interval of 21 days.

Table 9: Observations of callus multiplication (CoJ 83).
(Avg. fresh weight in grams)

Media	Fresh wt. (gm) (7 days)	Fresh wt. (gm) (14 days)	Fresh wt. (gm) (21 days)	Fresh wt. (gm) (28 days)
CMM_1	0.75 ± 0.05	1.02 ± 0.08	1.27 ± 0.18	1.24 ± 0.01
CMM_2	0.92 ± 0.01	1.21 ± 0.12	1.49 ± 0.20	1.47 ± 0.04
CMM_3	1.20 ± 0.10	1.48 ± 0.09	1.75 ± 0.12	1.73 ± 0.02
CMM_4	1.08 ± 0.07	1.44 ± 0.40	1.69 ± 0.14	1.65 ± 0.06

Table 10: Observations of callus multiplication (CoJ 83).
(**Avg. dry weight in grams)**

Media	Dry wt. (gms) (7 days)	Dry wt. (gms) (14 days)	Dry wt. (gms) (21 days)	Dry wt. (gms) (28 days)
CMM_1	0.5 ± 0.6	0.90 ± 0.08	0.10 ± 0.04	0.08 ± 0.04
CMM_2	0.5 ± 0.21	0.11 ± 0.06	0.12 ± 0.02	0.10 ± 0.02
CMM_3	0.6 ± 0.04	0.14 ± 0.40	0.17 ± 0.11	0.15 ± 0.11
CMM_4	0.7 ± 0.03	0.13 ± .015	0.15 ± 0.06	0.14 ± 0.06

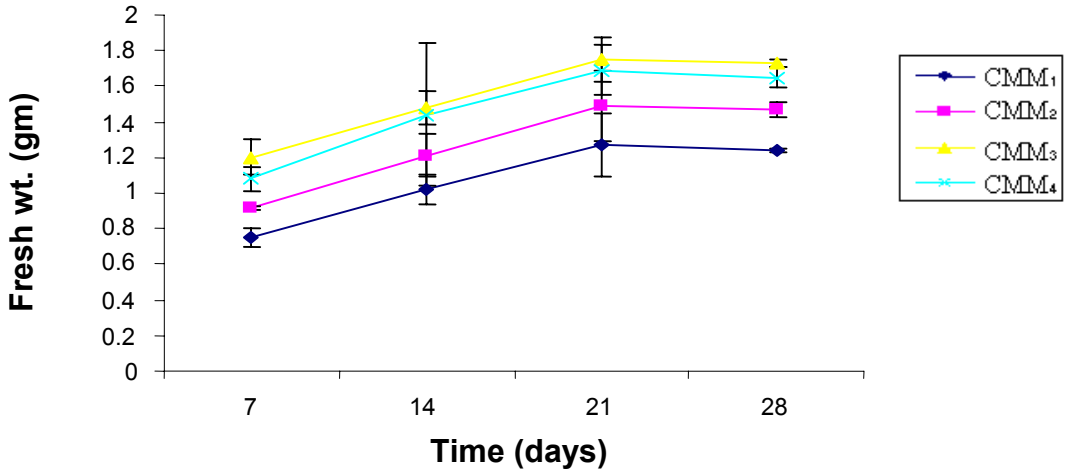


Figure: 6 Callus growth curve of (CoJ 83) on different media used.

4. g Regeneration from callus

Calli established on the media supplemented with 2, 4-D maintained and multiplied through subculturing for 1-2 cycle at 21 days each were segregated on the basis of physical appearance. The light creamish yellow, nodular calli were considered embryogenic, where as smooth, watery and whitish calli were considered to be non-embryogenic.

Gupta *et al*, 1995, reported four distinguishable types of calli. Callus type 1: organized, yellowish, nodular/globular embryoid containing compact callus; callus type 2: smooth, semi-organized, light yellowish, differentiated callus; callus type 3: spongy-type, soft, whitish, rough surface callus; callus type 4: unorganized, light yellowish, soft, rough surface callus. Callus type 3 and callus type 4 were unable to regenerate the plantlets. However callus type 1 and callus type 2 showed high potential for morphogenesis of sugarcane. Falco *et al,* 1999, reported that plant regeneration occurred only from the nodular and compact calli. We have also observed that regeneration was best observed from sectors of nodular embryogenic callus. It was also observed that in mixed sectors (compact/nodular and mucilaginous), some regeneration (thread followed by shoot like structures) occurs on early stages of subculturing but these sectors ultimately turned brownish or completely mucilaginous on repeated stages of subculturing.

The embryogenic calli was transferred to media containing only cytokinin or cytokinin and low level of auxin and kept in light. Four different media compositions based on MS salts were used to see the effect on shoot regeneration. RGM_1 (MS + 0.5 mg/l BAP), RGM_2 (MS + 0.5 mg/l BAP + 1 mg/l NAA), RGM_3 (MS + 0.5 mg/l BAP + 0.125 mg/l Kinetin + 0.5 mg/l 2, 4-D), RGM_4 (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin) with RGM 0(MS basal) taken as control. Ahloowalia and Maretzki, 1983, also reported that regeneration can be achieved in media with reduced conc. of 2,4-D.

The experiment was designed as the following, 5 different shoot regeneration medium were chosen, each with 10 culture jar and 1 cm^2 of embryogenic (compact, nodular) callus was inoculated in each bottle. Observation taken after 14 days.

Table 11: Observation on plant regeneration (10 bottles per replicate)

Media	Embryogenic callus (1 cm)^2 with plant regeneration(%)	
	CoS 8436	CoJ 83
RGM 0	-	-
RGM $_1$	58	55
RGM $_2$	65	62
RGM $_3$	47	51
RGM $_4$	74	68

Regeneration starts with thin thread like appearances emerging. Compact creamish white callus showed the appearance of threads. No regeneration was observed on RGM 0 (MS basal) media taken as control. Experimental observation showed that RGM_4 media supplemented with 0. 5 mg/l BAP and 0.5 mg/l Kn showed 74 % regeneration in CoS 8436 and 68% in CoJ 83. Cheema *et al*, 1995, reported transfer of plantlets differentiated from calli to MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l Kinetin for shoot proliferation. RGM_2 (MS + 0.5 mg/l BAP + 1 mg/l NAA), showed 65% regeneration in CoS 8436 and and 62% in CoJ 83 . Irwine *et al,* 1991, reported that combining NAA with Kinetin promoted rapid sugarcane regeneration from callus. It was also observed by Gupta *et al*, 1995, that MS

salts with 0.5 mg/l NAA+ 3.0mg/l Kn+30g/l sucrose+0.8% agarose produced 30-50 regenerated plantlets from proto calli.

Regeneration was lower in RGM_1 and RGM_3 media. In RGM_1 (MS + 0.5 mg/l BAP) 58% regeneration occurred in CoS 8436 and 55% in CoJ83. Gill *et al,* 2004, reported that higher shoot regeneration was achieved on a medium containing only cytokinin, BAP (0.5 mg/l). RGM_3 media supplemented with 0.5 mg/l BAP + 0.125 mg/l Kinetin + 0.5 mg/l 2, 4-D showed 47 % regeneration in CoJ 83 and 51% in CoS 8436. Chen *et al* (1988) reported plant regeneration from leaf explants when placed on a medium containing 1 mg/dm^3 2, 4-D.

The callus region showing regeneration turned greenish after kept in light. Fitch and Moore, 1990, reported that long-term regeneration of sugarcane callus cultures was maintained by selection of green callus on MS agar medium containing 0.5 mg/l picloram or 2, 4-D. Green callus on picloram media always regenerated into green plants. Histological studies have revealed that plant regeneration from sugarcane tissues occur by organogenesis (Liu and Chen, 1974) or somatic embryogenesis (Ho and Vasil, 1983a, b). Gill *et al,* 2004, reported that callus formation in sugarcane is explant, genotype and hormone dependent. Supplementation of the control medium independently with maltose (30 mg /l), proline (560 mg/l), abcissic acid (2mg/l) and reduced auxin i.e., 2, 4- D (3 mg/l) enhanced somatic embryogenesis considerably. Similarly, higher shoot regeneration was achieved on a medium containing only cytokinin, BAP (0.5 mg/l). We have also observed that regeneration was promoted in media supplemented with cytokinin (best regeneration is observed when BAP and Kn is combined). Chen *et al*, 1988, reported plant regeneration from leaf explants when placed on a medium containing 1 mg/dm^3 2, 4 D in continuous light.

From an area of one cm^2 of callus, initially 3-4 shoots of height 3-3.5 cm were formed within 14 days incubated in light. These shoots attained height of av. 4.5-6 cm in 18-20 days when transferred into multiplication medium and the no. of shoots increased gradually. Thus shoots were transferred to three different multiplication media: SM_1 (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin), SM_2 (MS + 0.2 mg/l BAP + 1.0 mg/l Kinetin) and SM_3 (MS+ 1.0 mg/l BAP +

1.5 mg/l Kinetin). The experiment was designed as the following, 3 different shoot multiplication medium were chosen, each with 3-culture jar. Observation was taken after 21 days.

Table 10: Observation of different media combination for multiplication

Media	No. of shoots/callus (1 cm^2)		Average height of shoots (cm)	
	CoS 8436	**CoJ 83**	**CoS 8436**	**CoJ 83**
SM_1	6.2 ± 0.02	5.5 ± 0.67	5.8 ± 0.50	5.6 ± 0.30
SM_2	7.25 ± 0.37	6.5 ± 0.8	5.2 ± 0.31	4.8 ± 0.91
SM_3	9.0 ± 0.1	8 .12± 0.5	4.80 ± 0.30	4.6 ± 0.42

The response shown by regenerated shoots in multiplication medium was similar to the response shown by explants inoculated in multiplication medium in light. Maximum shoot proliferation was observed in SM_3 media (MS+1.0 mg/l BAP and 1.5 mg/l Kn) with 9 shoots/callus in CoS 8436 and 8.12 shoots/callus in CoJ 83. Whereas highest shoot elongation was observed in SM_1 media containing MS + 0.5 mg/l BAP and 0.5 mg/l kn with 5.8 cm average height in CoS 8436 and 5.6 cm height in CoJ 83. Simultaneous rooting was also observed in the elongated shoots in all the mediums but these were very thin and week. Hence, it was necessary to further incubation in a rooting medium,

4. h Root development

Roots were induced on transferring the regenerated shoots to best rooting combination containing MS supplemented with NAA (5 mg/l) and sugar (7%). The elongated shoots were transferred to a medium lacking cytokinins as rooting does not occur on cytokinin rich medium. Higher level of sugar was necessary for root induction. Cheema *et al,* 1995, demonstrated rooting on MS medium supplemented with 7% sugar and 5mg/l NAA. There were about 16± 0.5 roots/shoot in CoS 8436 and average root length was about 2.2 cm± 0.48. In CoJ 83, an average of 12± 1.00 roots/shoots were obtained with an average root length (cm) of 2.0± 0.8. The observation was

taken after 14 days. Nadar and Heinz, 1977, reported that preferred auxin for root initiation was NAA. Rooting was induced in clumps.

Table 11: Observations of rooting in different media

Media	Avg. ht of 5 Shoots/clump		No. of shoots rooted/clump		Avg. root length (cm)	
	CoS 8436	CoJ 83	CoS 8436	CoJ 83	CoS 8436	CoJ 83
SRM_2	7.0 ± 0.70	6.2 ± 0.20	16 ± 0.50	12 ± 1.00	2.4 ± 0.48	2.0 ± 0.80

-

4.i Transplantation & hardening

After 10-15 days of culture on MS medium meant for rooting, the sufficiently rooted plantlets were transplanted to small trays for hardening prior to their final transfer to the soil. The rooted clumps/plantlets were gently removed from the culture vessel keeping the roots intact by using forceps with extreme care to avoid any mechanical damage and further the plantlets were separated from the shoot clumps. Plantlets were thoroughly washed with in tap water to remove any remaining medium possibly on them. Further plantlets were given Bavistin treatment (1gm/l) for 10 minutes to protect the plant from any near future fungal attack. Regenerated shoots were planted in hykotray containing soil and agropeat in 1:4(v/v). Plants were thoroughly watered and kept in poly house under humidity range of 75-85% and temperature of 26-28 ^{0} C for about 2-3 week. These plantlets were then transferred to shade house (75%) in which they would be kept under the humidity range of 60-70% with intermittent sprinkling of water for appox 2 weeks. The plants are then transferred to open nursery for final stage of acclimatization for another 2 weeks. When the plants have become well acclimatized they are given fertilizer treatment. Success of hardening protocol was determined by calculating survival %. Plants were ready for field transplantation after 6-7 weeks.

5. Summary and Conclusion

CoS 8436 and CoJ 83 are important varieties of sugarcane cultivated in Punjab. In the present study we have tried to optimize and improve the mass propagation protocol of these two varieties and study their nutritional aspects for callus culture and regeneration.

1. Following sterilization protocol was found satisfactory for the surface sterilization of sugarcane explants

Washing of spindle under running tap water for 30 minutes.

Explants were soaked in an aqueous solution containing 0.1 % Bavistin (BASF, India Limited) and few drops of Tween 20 for 20 minutes. Explants were again washed under running tap water for 10 minutes.

- They were then taken inside the laminar hood for further sterilization. Inside the laminar hood, sterilization with savlon (Johnson & Johnson) and 0.1% mercuric chloride (Ranbaxy) for 7-8 minutes each is carried out with intermittent washing with autoclaved water.

2. Cytokinins were found to play an important role in axillary bud initiation. It was observed that media S_1M_1 (MS + 0.5 mg/l BAP + 0.5 mg/l Kn) gave the best results of bud break 95% in CoS 8436 and 80% in CoJ 83. However S_1M_2 (1.0 mg/l BAP + 1.5 mg/l Kn) gave maximum no. of shoots/ explant 5.75 in CoS 8436 and 4.25 in CoJ 83.
3. At the multiplication stage, the two combinations SM_3 (MS+ BAP 1.0 mg/l + 1.5 mg/l Kinetin) and SM_2 (MS+ BAP 0.2 mg/l + 1.0 mg/l Kinetin) gave best results for shoot proliferation. Maximum shoot proliferation was obtained in the SM_3 medium with a multiplication rate of 4.0 in CoS 8436 and 3.63 in CoJ 83 whereas multiplication rate in SM_2 is 3.10 for CoS 8436 and 2.93 for CoJ 83. However at this stage rate of increase in shoot height been found to be maximum in SM_1 (MS + 0.5 mg/l BAP + 0.5 mg/l Kinetin).

4. It was observed that rooting occurred in clumps. Sugar and auxin played an important role in rooting. SRM_2 (MS + 5mg/l NAA+ Sugar 70 gm/l) showed better response for rooting then SRM_1 (MS + 3mg/l NAA+ Sugar 30 gm/l). Root initiation was noted after 5 days and within 12 days profuse rooting was observed in SRM_2 medium. In SRM_2 an average of 12±1.99 roots/shoots were obtained CoS 8436 with an average root length (cm) of 2.0±0.09 and in CoJ 83 an average of 10± 2.0 roots/shoot clumps were obtained with an average root length 1.4±0.04 (cm) of shoots rooted in clumps. Rooted plats were transplanted in the hykoytrays with a potting mixture of agropeat and soil in 1:4 ratios.
5. Callus cultures were established by subculturing spindle leaf of 2.0-2.5 cm in size on MS medium supplemented with different concentration of auxin (2, 4-D). CoS 8436 exhibited rapid callusing within 8-10 days, whereas CoJ 83 took 12-14 days for callus induction. %age of callus induction was better in MS medium supplemented with concentration of 2, 4-D 3 mg/l (CIM_3) and 2, 4-D 4 mg/l (CIM_4). Good callus induction 85% in CoS 8436 and 78% in CoJ 83 was obtained in CIM_3, whereas 66% induction in CoS 8436 and 72% was obtained in CIM_4. A mucilaginous, nodular calli was first to develop. Subsequently a creamish white compact morphogenic callus developed over the mucilaginous tissue. Callus multiplication was carried on reduced concentration of 2, 4-D. Good callus proliferation was obtained in CMM_3 (MS + 1.5 mg/l 2, 4-D) and CMM_4 (MS + 2 mg/l 2, 4-D).
6. Regeneration was observed only in the sectors, which were creamish, white, compact and nodular. Regeneration from calli was obtained after transferring the callus to a medium containing only cytokinin or low concentration of auxin. Two regeneration media, RGM_4 (0.5 mg/l BAP + 0.5 mg/l Kn) and RGM_2 (MS + 0.5 mg/l BAP + 1 mg/l NAA) were found to give satisfactory results for plant regeneration. RGM_4 media supplemented with 0. 5 mg/l BAP and 0.5 mg/l Kn showed 74 % regeneration in CoS 8436 and 68% in CoJ 83. RGM_2 (MS + 0.5 mg/l BAP + 1 mg/l NAA), showed 65% regeneration in CoS 8436 and 62% in

CoJ 83. The regenerated shoots were then transferred to shoot multiplication medium. Maximum shoot proliferation was observed in SM_3 media (MS+1.0 mg/l BAP and 1.5 mg/l Kn) with 9 shoots/callus in CoS 8436 and 8.12 shoots/callus in CoJ 83. Whereas highest shoot elongation was observed in SM_1 media containing MS + 0.5 mg/l BAP and 0.5 mg/l Kn with 5.8 cm average height in CoS 8436 and 5.6 cm height in CoJ 83. For rooting, clump was transferred to SRM_2 media in which MS was supplemented with 5 mg/l NAA and 70 gm/ l of sugar and an average of 16 shoots rooted/ clump in CoS 8436 and 12 shoots rooted/ clump in CoJ 83 were obtained. Transplantation was done by using soil and agrpopeat mixture in 1:4 ratio in hykotrays. These trays were then kept inside the polyhouse for two weeks at Tissue culture facility at TIFAC CORE followed by placing them in the shade house for two week and then in open area for one week for the proper hardening of the plants.

6. Bibliography

- Aftab, F; Zafar, Y; Malik, K.A and Iqbal, I (1996). Plant regeneration from embryogenic cell suspensions and protoplasts in sugarcane (Saccharum spp. hybrid cv. CoL-54). Plant Cell Tissue and Organ Culture, 44(1): 71 – 78.
- Ahloowalia, B.S. and Maretzki, A. (1983). Plant regeneration via somatic embryogenesis in Sugarcane. Plant Cell Reports, 2(1): 21-25. *
- Ali, Kazim and Shahid Afghan (2001). Rapid multiplication of sugarcane through micropropagation technique. Pakistan Sugar Journal, 16(6): 11-14, 14 ref.
- Anita, P.; Jain, R.K.; Sehrawat, A.R and. Punia, A. (2000). Efficient and cost- effective micropropagation of two early maturing varieties of sugarcane (*Saccharum* spp.). Indian Sugar, 50: 611-618. *
- Armstrong, C.L. and Green, C.E. (1985). Establishment and maintenance of friable, embryonic maize callus and the involvement of L-proline. Planta, 164: 207-14. *
- Arencibia, Ariel.; Vazquez, R.I.; Prieto, D.L.; Tellez, P; Carmona, E.R.; Coego, A.; Hernandez, L. ; De la Riva,G.A.; Selman-Housein, G.(1997).Transgenic sugarcane plants resistant to stem borer attack. Mol. Breed., 3: 247-255.
- Barber (1931). History and development of sugarcane as a world crop in *The Sugarcane:* 1-5.
- Baksha, R., Alam R; Karim, M.Z; Mannan, Sk; Podder, B.P and Rahman, A.B.M.M. (2003). Effect of Auxin, Sucrose and pH Level on *in vitro* Rooting of Callus Induced micro Shoots of Sugarcane (*Saccharum officinarum).* Journal of Biological Sciences, 3 (10): 915-920.
- Blanco, M.D.A.; Nieves, N.; Sanchez, M.; Borrto, C.G.; Castillo, R.; Gonzalez (1997). Protein changes associated with plant regeneration in embryogenic calli of sugarcane. Plant Cell Tissue and Organ Culture, 51: 153-158. *

- Bower, R.; Birch, R.G. (1992). Transgenic sugarcane plants via microprojectile bombardment. Plant Journal, 2: 409-416. *
- Brandes (1956). History and development of sugarcane as a world crop in *The Sugarcane:* 1-5.
- Bright, S. W. J.; Jones, M. G. K. (1985). Cereal tissue and cell culture. Martinus Nijhoff; Dr. W.Junk G.V. publishers, Correct, Boston. Lancaster*
- Brisibe, E.A.; Miyake, H.; Taniguchi, T.; Maeda, E. (1994). Regulation of somatic embryogenesis in long term callus cultures of sugarcane. New Phytology, 126 : 201-307. *
- Burner, D.M. and Grisham, M. P. (1995). Induction and stability of phenotypic variation in sugarcane as affected by propagation procedure. Crop Science, 35(3): 75-880, 40 ref. *
- Cheema, A.S.; Singh, H and Gosal. S. S (1992). Response of different genotypes to callus induction and plant regeneration in sugarcane. Crop Improvement, 19: 6-13.
- Cheema, Ravinder Kaur; Gupta, J. N (1995). Plants regenerated from protoplasts of sugarcane (*Saccharum officinarum*). Current Science, 68(6): 650-653, 23 ref.
- Chen, W. H.; Davey, M. R; Power, J. B.; Cocking E. C.; Davey, M. R.; Sotak, R.; Gartland, J. S.; Mulligan, B. J (1987). Transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene. Plant Cell Reports, 6(4): 297-301.
- Chen, W. H.; Davey, M. R; Power, J. B. and E. C. Cocking (1988). Control and maintenance of plant regeneration in sugarcane callus cultures. Journal of Experimental Botany, 39: 251-261. *
- Chen, W. H.; Davey, M. R; Power, J. B. and E. C. Cocking (1998). Sugarcane protoplasts: factors affecting division and plant regeneration. Plant Cell Reports, 7(5): 344 – 347. *
- Chowdhury, M.K.U.; Vasil, Indra K (1992). Stably transformed herbicide resistant callus of sugarcane via microprojectile bombardment of cell

suspension cultures and electroporation of protoplasts. Plant Cell, 11(10): 494-498.

- Chowdhury MKU and Vasil IK (1993). Molecular analysis of plants regenerated from embryogenic cultures of hybrid sugarcane cultivars (*Saccharum* spp). Theor Appl Genet, 86: 181-188. *
- Dhumale, D.B; Ingole, G. L.; Durge, D.V. (1994). In vitro regeneration of sugarcane by tissue culture. Annals of Plant Physiology, 8(2): 192-194, 5 ref. *
- Deerr, N. (1949). The history of sugar. Chapman and Hall Ltd, London 1:258 pp.
- Enriquez-Obregon, G. A.; Vazquez-Padron. (1998). Herbicide resistant sugarcane plants by Agrobacterium mediated transformation. Planta, 206: 20-27.
- Falco, M.C.; Neto, A.T.; Mendes, B. M. J.; Arias, F.J.Z. (1996). Isolation and cultivation of protoplasts. Brasileira de Fisiologia Vegetal, 8(3): 175-179; 25 ref.
- Falco, M. C.; Mendes, Beatriz M. Januzzi; Neto, Augusto Tulmann and da Glória (1999). Histological characterization of In vitro regeneration of Saccharum sp. Centro de Energia Nuclear na Agricultura/USP, CP 96, Piracicaba, SP, 13416-970, Brazil.
- Falco, M. C.; Neto, A. T.; Ulian, E. C. (2000). Transformation and expression of a gene for herbicide resistance in Brazilian sugarcane. Plant Cell Reports, 19:12:1188-1194, 31 ref.
- Fitch, M; Irvine, J.E.; and P.H. Moore (1983). The induction of callus in sugarcane tissue cultures by selected chemicals. Plant Cell, Tissue and Organ Culture 2(2): 141-149. *
- Fitch, M.; and Moore, Paul H. (1990). Comparison of 2, 4-D and picloram for selection of long-term totipotent green callus cultures. Plant Cell, Tissue and Organ Culture 20(3):157 -163. *

- Fitch, M.; Moore, Paul H. (1993). Long-term culture of embryogenic sugarcane callus. Plant Cell, Tissue and Organ Culture 32(3): 335-343.*
- Gallo-Meagher, M.; Irvine, J.E. (1996). Herbicide resistant transgenic sugarcane plants containing the bar gene. Crop Sci.36:1367-1374. *
- Gallo-Meagher, M., English, R. G. and Abouzid, A. (2000). Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. In Vitro Cell Dev. Biol. Plant, 36: 37–40. *
- Geetha, S.; Padmanabhan, D (2001). Effect of hormones on direct somatic embryogenesis in sugarcane. Sugar Tech., 3(3): 120-121; 8 ref.
- George, E.F. and P.D. Sherrington, (1984). Plant propagation by tissue culture: handbook and directory of commercial laboratories. Exegetics, Bassingstokes., Ed 2, pp: 71-83.
- Gill, Navdeep K; Gill, R.; Gosal, S.S (2004). Factors enhancing somatic embryogenesis and plant regeneration in sugarcane *(Saccharum officinarum L.)*.Indian Journal of Biotechnology 3: 119-123.
- Gupta, J.N; Kaur, Ravinder; Cheema, G.S. (1995). Plants regenerated from protoplasts of sugarcane. Current Science, 68 (6): 650-653.
- Hagelberg, G.B. (1985). Sugar in the Caribbean: turning sunshine into money. The Woodrow Wilson International Center for Scholars, Washington, D.C. 42 pp. *
- He, D. G.; Tanner, G., and Scott, K. J., (1986). Establishment and characterization of long-term embryogenic maize callus and cell suspension cultures. Plant Science, 45: 111-17.
- Hendre, R.R.,;Fyer, R.S.; Kotwal, M.; Khuspe, S.S. and Mascarenhas, A.F. (1983). Rapid multiplication of sugarcane. Sugarcane 1: 5-8. *
- Heinz, DJ; Mee, GWP (1969). Plant differentiation from callus tissue of saccharum species .Crop Sci 9: 346-349. *
- Heinz, DJ; Nickell; LG; D.J.; Krishnamurthi, M; Maretzki, A. (1977). Cell Tissue and Organ Culture in sugarcane improvement. In applied and

fundamental aspects of Plant Cell Tissue and Organ Culture. Eds J. Reinert and Y.P.S. Bajaj. Springer- Verlag, Berlin. pp. 3-17.

- Heyser, J.W.; Dykes, T. A.; De Mott, K.J.; Nabors, M. W. (1983**).** High frequency long term regeneration of rice from callus culture. Plant Science Letters, 29:175-182.
- Ho, W.J.; and Vasil, I.K., (1983a). Somatic embryogenesis in sugarcane (*Saccharum officinarum L.).* The morphology and physiology of callus formation and the ontogeny of somatic embryos. Protoplasma, 118:169-80. *
- Ho, W.J. and Vasil, I.K. (1983b) Somatic embryogenesis in sugarcane *(Saccharum officinarum L.)*. Growth and plant regeneration from embryogenic cell suspension cultures. Annals of Botany, 51: 719-26.
- Hossain, M.A.; Begum, S.; Miah, M.A.S.; Uddin, M.J. and Miah**,** A.J. (1996). Induction of callus and regeneration of plants in Sugarcane. In: Plant Tissue Culture. A. S. Islam (ed.), pp: 76-79. Oxford & IBH Pub. Co. Pvt. Ltd. New Delhi.
- Hoy, J.W.; Bischoff, K. P.; Milligan, S. B.; Gravois, K. A. (2003). Effect of tissue culture explant source on sugarcane yield components. Euphytica, 129(2): 237-240.
- Irwine, J.E.; Benda, G. T. A.; Legendre, B.L.; Machado, G.R. (1991). The frequency of marker changes in sugarcane plants regenerated from callus culture. Evidence for vegetative and genetic transmission, epigenetic effects and chimerial disruption. Plant Cell Tissue and Organ Culture, 26:115-125
- Ingelbrecht, I. L.; Irwine. J. E.; Mirkov, T.E. (1999). Post transcriptional gene silencing in Transgenic sugarcane. Dissection of homology-dependent virus resistant in a monocot that has a complex polyploidy genome. Plant Physiology, 119: 1187-1197. *
- Islam, R.; Haider, S. A.; Alam, M. A.; Joarder, O. I. (1996). High frequency somatic embryogenesis and plant regeneration in sugarcane. Rice Biotechnology Quarterly, 25.

- Jenkins, G.H. (1966). Introduction to cane sugar technology. Elsevier Publishing Co., New York. 478 pp.
- Kaur, Ajinder; Gosal, S. S.; Raman Gill; Thind, K. S. (2001). Induction of plant regeneration and somaclonal variation for some agronomic traits in sugarcane *(Saccharum officinarum L.).* Crop Improvement, 28(2): 167-172; 6 ref.
- Lal, N and Singh, H.N. (1994). Rapid clonal multiplication of sugarcane through tissue culture. Plant Tissue Culture, 4: 1-7.
- Lee (1987). Micropropagation of sugarcane (Saccharum spp.) Plant Cell, Tissue and Organ Culture 10(1): 47-55.
- Leal, M. R.; Maribona, R. H.; Ruiz, A.; Korneva, S.; Canales, E.; Dinkova, T. D.; Izquierdo, F.; Coto O. and Rizo, D. (1996). Somaclonal variation as a source of resistances to eyespot disease of Sugarcane. Plant Breeding 115: 37-42. *
- Liu, M.C. and Chen, W. H. (1974). Histological studies on the origin and process of plantlet differentiation in sugarcane callus mass. Proceedings International society for Sugarcane Technology (South Africa),15: 115-28.
- Liu, M.C. (1984). Sugarcane. In: Handbook of plant cell culture, Vol II. Sharp, W.R., Evans, O.A., Amirato, P.V. and Yamada, Y. (Ed). Macmillan Publishing Co., New York 224 pp.
- Liu, M. C. (1993). Factors affecting induction, somatic embryogenesis and plant regeneration of callus from cultured immature inflorescence of sugarcane. Journal of Plant Physiology, 141:714–720.
- Lorenzo, Jose Carlos ; María de los Angeles; Peláez, Osvaldo ; González, Alfredo ; Cid, Mariela ; Iglesias ,A. ; González ,Boris; Escalona, Maritza; Espinosa, Patricia; Borroto (2001). Sugarcane micropropagation and phenolic excretion. Plant Cell, Tissue and Organ Culture, 65(1):1-8.
- Lorenzo; José Carlos, González; Boris Luis, Escalona; Maritza, Teisson; Claude and Borroto (1998). Sugarcane shoot formation in an improved

temporary immersion system. Plant Cell, Tissue and Organ Culture, 54(3):197-200.

- Lowe, K.; Taylor, D.B.; Ryan, P. and Paterson, K.E. (1985). Plant regeneration via organogenesis and embryogenesis in the maize inbred line B78. Plant Science, 41:125-32.
- Mamun, M.A; Sikdar, M.B.H.; Paul, Dipak Kumar, Rahman, M.; Mizanur (2004). In vitro Micropropagation of some Important Sugarcane Varieties of Bangladesh.Asian Journal of Plant Sciences. 3 (6): 666-669.
- Mulleegadoo, K. D.; Dookun, A. (1999). Effect of explant source and genotype on growth of sugar cane In vitro Revue Agricole et Sucrière de l'Île Maurice, 78(2):35- 39; 18 ref. *
- Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and biossays with tobacco cultures. Physiology Plantarum, 15: 473-497.
- Nabors, M. W., (1982). Long term plant regeneration, somatic embryogenesis and green spot formation in secondary oat callus, 107:153-166. *
- Nabors,M.W.; Heyser, J.W.; Dykes, T.A. and Demott, K.J.(1983). Long-duration, high-frequency plant regeneration from cereal tissue cultures. Planta, 157:385-91.
- Nadar, H.M. and Heinz, D.J. (1977). Root and shoot development from sugarcane callus tissue. Crop Science, 17: 814-816. *
- Naritoom, K.; Sooksathan, K.; Korpradittskul, V.; Suksathan, I.; Visessuwan, R.; Burikam, S.; Klingkong, S.; Suriyachaiyakorn, M.(1993). Plant regeneration from shoot tip culture of sugarcane. Kasetsart Journal of Natural Sciences, 27: 286-291.*
- Nickell; LG (1964). Tissue and cell culture of sugarcane another research tool. Hawaii Plant Research, 57:223-229. *
- Noguera, A., E. Diaz, N. Paz and J. Ramallo, 2002.Sugarcane: micropropagation of recalcitrant varieties. Advance Agroindustrial, 23: 25-27. *

- Oropeza, M and E.D, Garcia.(1996). Somaclonal variants resistant to sugarcane mosaic virus and their agronomic characterization. In Vito Cell. Dev. Biol. Plant, 34:267-275. *
- Oropeza, M and E.D, Garcia; Guevara, P(1995). Identification of somaclonal variants of sugarcane resistant to sugarcane mosaic virus via RAPD markrs. Plant Mol. Biol.Rep., 13:182-191. *
- Ozias-Akins, P.; and Lorz, H (1984). Progress and limitations in the culture of cereal protoplasts. Trends in Biotechnology, 2:119-23. *
- Patel, A.A.; Patel, S.R; Patel, C.L. and Prajapati, B.S. (2000). Regeneration of tissue culture plantlets through callus culture in sugarcane cultivar CoC-671. Journal of Genetics and Plant breeding, 60(2): 255-257, 8 ref. *
- Patel, A.A.; Patel, S.R; Patel, C.L. and Prajapati, B.S. (2001). Effect of media composition on *in vitro* multiplication of sugarcane varieties. Ind. J. Gen. Pl. Breed., 61: 82-83. *
- Prajapati, B. S.; Patel, C. L.; Patel, S. R.; Patel, A.A (2000). A Regeneration of tissue culture plantlets through callus culture in sugarcane cultivar CoC 671. Indian Journal of Genetics & Plant Breeding, 60(2): 255-257; 8 ref.
- Pawar, S. V.; Patil, S. C.; Jambhale, V. M.; Naik, R. M.; Mehetre, S. S (2002).Rapid multiplication of commercial sugarcane varieties through tissue culture Indian Sugar, 52(3):183-186; 9 ref. *
- Purseglove, J.W. (1979). Tropical crops: monocotyledons. Longman Group Ltd., London. 607 pp. *
- Rodríguez, S.; Mondéjar, C.; Ramos, M. E.; Díaz, E.; Maribona, R.; Ancheta, O.; (1996). Sugarcane somatic embryogenesis: a scanning electron microscopy study. Tissue & Cell, 28(2): 149-154, 26 ref. *
- Sargent, H. R.; Godwin, I. D.; Adkins, S. W. (2000). The effect of exogenous polyamines on somatic embryogenesis and plant regeneration from *Sorghum bicolor* and *Saccharum* spp. Plant Cell Reports, 19(12): 1188-1194, 31 ref. *

- Shenk, R.U. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., 50:199-204. *
- Singh, B.; Yadav, G. C.; Lal, M. (2001). An efficient protocol for micropropagation of sugarcane using shoot tip explants. Sugar Tech, 3(3):113-116; 10 ref. *
- Skoog, F. and C.O. Miller (1957). Chemical regulation of growth and organ formations in plant tissue cultures *in vitro*. Symp.Soc. Exp. Biol., 11: 118-131.
- Taylor, Paul W. J. ; Ko, Hian-Lien; Adkin, Stephen W. ; Rathus, Carl; Birch, Robert G. (1992). Establishment of embryogenic callus and high protoplast yielding suspension cultures of sugarcane (Saccharum spp. hybrids). Plant Cell, Tissue and Organ Culture, 28(1): 69-78.
- Thorpe, TA (1986). Organogenesis in vitro: structural, physiological and biochemical aspects. Int. Rev. cytol, suppl. 11 A: 71-112. *
- U.C. Prinsen Geerligs, The World Cane Sugar Industry, Past and Present, 1912, Norman Rodger Altrincham, Manchester, England.
- Vasil, I. K. (1983a). Regeneration of plants from single cells of cereals and grasses. In genetic engineering in eukaryotes. Eds P.F. Lurquin and A. Kleinhofs. Plenum Press, New York and London. Pp. 233-52. *
- Vasil, I. K. (1983b). Isolation and culture of protoplasts of grasses. International review of cytology, Supplement, 14:79-88. *
- Vasil, I. K. (1987) Developing cell and tissue culture systems for the improvement of cereals and grass crops. Journal of Plant Physiology, 128:192–218. *
- Vasil, I. K. and Lu, C (1984). Somatic embryogenesis in long term callus cultures of *Zea mays L*. (Gramineae). American Journal of Botany, 71: 158-61. *
- Wagley, LM; Gladfelter, H.J; Phillips, G.C (1987). De Novo shoot organogenesis of *Pinus eldarica*, *In vitro*. Macro and micro-

photographic evidence of De Novo regeneration. Plant Cell reports, 6: 167-171. *

- Wrigley, G. 1982. Tropical agriculture: the development of production. Fourth ed. Longman Inc., New York. 496 pp.
- http://www.agricoop.nic.in
- *Abstracts reviewed only