TISSUE CULTURE STUDIES OF SUGARCANE

A

DISSERTATION

By

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ABSTRACT

Sugarcane assumes the important position in the economy of India by contributing nearly 1.9% of National GDP. Sugarcane is cultivated in over 4 million hectares spreading over a wide range of agro-ecological situations. Sugarcane farmers are facing serious problems such as pest and disease infestation especially red rot, non-availability of good seed material, support from industry etc. It is need for the hour to find out and implement technological interventions for the same. Plant Tissue culture is a tool for obtaining rapid, mass multiplication of disease free, true to type planting material. Mass propagation protocol once standardize, can be utilized for rapid multiplication of disease free stock material, which further be, multiplied in strict management conditions for bulking up. Plant Tissue culture can also be used for rapid dissemination of newly released varieties with important agronomic characters.

In the present dissertation, we have tried to optimize the culture conditions of CoJ 85, which is an important variety in Punjab. Through our work, we have found that MS Basal + 0.5 mg/lt BAP + 0.5 mg/lt Kn + 30 gm/lt Sugar + 8.0 gm/lt agar is the best among the different combinations for the initiation of this variety. For the multiplication stage, best multiplication rate is observed in MS Basal + 1.0 mg/lt BAP + 1.5 mg/lt Kn + 30 gm/lt. We have also found that MS + 5 mg/lt NAA + 70 gm/lt sugar responded significantly for the rooting and for callus formation, MS + 2,4-D (4mg/l) was found to be the best for the callus induction.

CANDIDATE'S DECLARATION

Myself, Rupinder Singh, hereby declare that the work presented in the dissertation entitled, **"TISSUE CULTURE STUDIES OF SUGARCANE"** for 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, Punjab, India, is an authentic record of my own work during the period of five months from January 2003 to May 2003, under the supervision of Mr. Dipal Roy Choudhury, Research Scientist, TIFAC, CORE. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

Place: Patiala
Date:

(RUPINDER SINGH)

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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CONTENTS

1.0 INTRODUCTION

Plant tissue culture (PTC) is a generic description which embraces plant protoplast, plant cell, plant tissue, plant organ and plant culture, where these various types of culture involve, as a common factor, the growth of microbe-free plant material in an aseptic (sterile) environment, such as sterilized nutrient medium in a test tube. Basically it is the branch of biotechnology, which is used to clone plants at a very high speed without the restriction of the season (Bhojwani and Razdan, 1983). It involves culture of plant parts or tissues or organs aseptically in a proper container where the environment and the nutrition can be rigidly controlled. All plant cells retain the ability to use all of their genes and thereby can produce any type of tissue and eventually whole plants. This ability to generate any cells from such starting tissue is called Cellular "totipotency".

The technique of plant tissue culture may play a key role in the “Second Green Revolution’’ in which biotechnology and gene modification are being used to improve crop yield and quality. Usually, the plant part (explant) is placed in a suitable tissue culture media, proliferation of the lateral buds or adventitious shoots or the differentiation of the shoots results in tremendous increase in the number of shoots available for rooting. This process is known as micropropagation. It offers several distinct advantages that are not possible with conventional propagation techniques.

- Through micropropagation, it is possible to produce over a million clonally uniform plants, within one year in vitro from a single explant (shoot tip, nodal segment, leaf pieces etc), e.g. Eucalyptus.
- Through micropropagation, newly developed cultivars can also be released for commercial planting within a short period of time.
- Micropropagation can also be used for production of disease/pathogen free stock material (widely used in Horticulture industry) - as thorough meristem culture.

Apart from these conventional benefits, research on various aspects has widened the scope of Plant tissue culture.

- Secondary metabolite production through Tissue culture technology has been proved more efficient as compared to conventional extraction from field grown/naturally cultivated plants - commercial production of the napthoquinone pigment Shikonin (Bhau, Brijmohan Singh,1999).

- Plant tissue culture has also been used in the production of flavours, sweeteners, natural colourants and bio-pharmaceuticals (Khanna, P. 1982).

- Regeneration of the plants from callus is found to be an important source of variation (Skirvin and Janick, 1976), This phenomenon is called as Somaclonal variation which can also be used for the creation of new plant varieties with increased importance (Evans and Sharp, 1986). The origin and expression of the observed variations are usually very diverse, but the occurrences of stable gene mutations have been proved in many species (McCoy and Phillips, 1982). One of the more frequent types of variation is a difference in chromosomal number, e.g. aneuploidy, polyploidy, mixoploidy. This may be due to many reasons such as source of explant used or effects of culture process itself (lengthy culture periods, growth regulators used, nutritional stress etc) leading to changes in chromosome no/position. All these variations are found to increase with increase in culture periods. These changes may be either permanent (genetic) or temporary (epigenetic) in nature.

Tissue culture also found wide application in the fields of forestry and bio-diversity conservation. Mass production of plantlets for afforestation programme depends greatly in advancement of tissue culture techniques. Tissue culture also plays an important role in the preservation and conservation of the endangered plants.

Even though tissue culture has tremendous applications in various fields, it has some limitations also. Tissue culture requires advanced manpower skill, specialized equipments and capital intensive facilities. Tissue culture techniques are usually specific and hence success rate varies widely.

1.a. History of Tissue Culture

The concept of plant tissue and cell culture was conceived as early as 1902 by the German botanist Gottlieb Harberlandt and published a paper entitled " Experiments on the culture of isolated cells". It was only during the last three decades or so, that its application have increased tremendously both in the basic research as well as applied fields especially in the agriculture and horticulture. Haberlandt had attempted to culture chlorophyll-containing cells and demonstrate the totipotency of cells. Though his success was not enthusiastically received during his time, he did manage to initiate a new method of plant propagation, which has become known as ' Plant Tissue Culture' . Since 1952, there has been an increasing interest in plant tissue culture for propagating many types of plants. Meristem tip culture has been primarily used to increase stock of desired plants that do not reproduce true through sexual means. Skoog and Miller (1957) gave the concept of hormonal control of organ formation. According to them, combination of auxin and cytokinin in tissue culture media determines differentiation of roots and shoots. High level of auxin in the medium induces rhizogenesis vis a vis high level of cytokinin promotes caulogenesis. Single cell culture became possible after the work of Muir *et al* (1954), who developed the nurse culture technique. E.C Cocking isolated protoplast for the first time, however; totipotency of isolated tobacco protoplast for regeneration into intact plantlet was demonstrated by Takebe *et al* (1971). Another attempt was made by Carlson *et al* (1972) when first inter-specific somatic hybrids plants were produced by fusion of *Nicotiana longsdorffi* and *Nicotiana glaucca* protoplast. In early 1960's, Murashige completed a study while working in Skoog's lab leading to commercial application of tissue culture. Murashige had developed a culture medium especially for rapid growth and bioassay of tobacco callus (Murashige and Skoog 1962). Reports concerning the recovery of plants from haploid cells began to appear in 1960's and the first success was obtained with Datura (Guha and Maheshwari, 1960). Consequently, the technique of tissue and organ culture are used for rapid multiplication of plants for the biosynthesis of plant constituents, for genetic improvement of crops, for obtaining disease free clones and for preserving valuable germplasm.

At present, some 300-plant species have either been reported/successfully demonstrated to be produced through tissue culture (Vasil and Vasil, 1980). Of the various plant species, ornamentals have been most successfully multiplied through tissue culture technique on commercial scale. The most glaring example is of orchid, the trade of which has been revolutionized by tissue culture (Hartmann and Kester, 1975; Morel, 1975; Rao, 1977) and now a days successfully grown as a cottage industry in Thailand. Transgenic plants was developed by transformation of tobacco with Agrobacterium (Horsch *et al* 1984), a novel bacteria known for inducing callus and a new terminology, Genetic engineering had been coined. Development of biolistic gene transfer method for plant transformation (Sanford *et al 1990;* Klein *et al* 1987) has now been successfully used as an alternative to Agrobacterium mediated gene transfer.

1 b. Achievements of India in Plant Tissue Culture

In India, tissue culture was initially reported by Maheshwari and Rangaswamy in 1958 for the regeneration of somatic embryos in vitro from the nucellus of Citrus ovules. A novel technique of test-tube fertilization was developed in Delhi University to overcome incompatibility in plants that is exhibited in wide hybridisation. Another landmark research in plant breeding and genetics was the production of haploids through another culture of Datura (Guha and Maheshwari, 1964) achieved for the first time at Delhi University. Development of triploid plants through endosperm culture was also first developed from Delhi University in the 1970' s.The *in vitro* flowering of Bamboo, which is a rare phenomenon, was demonstrated by NCL scientists in the (Nadgauda, R.S., *et al*.).

A.V. Thomas & company was the first to put India on the modern tissue culture technology map of the world by setting up a commercial micropropagation unit in 1987 in Cochin Export processing Zone (Govil and Gupta, 2000). In the following year, three other companies has also started commercial laboratory, **1)** Indo American Hybrid Seeds (IAHS), Bangalore, 2**)** Hindustan Lever Ltd, Mumbai, 3**)** Unicorn Biotech, Hyderabad.

1.c. Benefits of Plant Tissue Culture

Plant tissue culture research is multi-dimensional and it has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry. The techniques include culture of cells, anthers, ovules and embryos on experimental to industrial scales, protoplast isolation and fusion, cell selection and meristem and bud culture. Instead of being a tool for rapid propagation, tissue culture became an alternating tool of breeding by selecting and testing for useful variants (Heinz *et al* 1971, Babra *et al* 1977, Morales *et al* 1989). The culture of the plant cells either as a callus tissue or as liquid suspension provides important techniques that can be preliminary to the regeneration of the whole plants because of the potential genetic variability associated with these systems. Spontaneously arising changes in tissue culture have been recognized as rich source of variability. In the Netherlands alone, over 100,000,000 plants are produced using micropropagation each year (Hall, 1999). Plant tissue culture is also advantageous to growers because the overwhelming number of plants can be produced using the tissue collected from a single parent plant and the plant which itself remains unharmed in the tissue harvesting process. Crop production through micropropagation also eliminates the possibility of any interruption in the growing season because it can be carried out inside the carefully regulated environment of a greenhouse. Because the chemical and physical environment inside a greenhouse can be closely monitored, any lull in production that might typically occur as a result of seasonal change can be avoided. (Lineberger, 2002).

Applications include:

- Micropropagation using meristem and shoot culture to produce large numbers of identical individuals
- Screening programmes of cells, rather than plants for advantageous characters
- Large-scale growth of plant cells in liquid culture as a source of secondary products
- Crossing distantly related species by protoplast fusion and regeneration of the novel hybrid
- Production of dihaploid plants from haploid cultures to achieve homozygous lines more rapidly in breeding programmes
- As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants
- Removal of viruses by propagation from meristematic tissues

SUGARCANE - AN OVERVIEW

1.d. History

The Sugarcane has been known from the earliest times, and is referred to in historical records going back into the remote days of ancient civilizations, which flourished long before the Christian era. Actual extraction of sugar from it does not appear to had been discovered until much later. The original home of *Saccharum officinarum* was in dispute until Brandes exhaustively surveyed the evidence and concluded that it was originated in New Guinea, where from ancient times, various forms of this thick, tall, tropical cane has been grown as a native domestic garden crop for chewing. Prior to this, Barber had discussed the subject under three headings: historical, linguistic and botanical.

The derivation of all names, for sugar and sugarcane from the Sanskrit "Shakkara" is adduced as linguistic evidence of Indian origin on the basis of the meaning 'a new crop from the east'. Barber concludes that botanical studies indicate two separate classes of cane with differing origins: the thin Indian indigenous canes of North India and tropical forms from space islands of Oceania, with New Guinea as a possible nucleus. Noel Deerr (1948) refers to South Pacific as being the source of origin and stated that *S. officinarum* is indigenous to that region. After much examination and discussion of available evidence by Brandes, his firm conclusion was that New Guinea is the undoubted home of the species and it represents the generally accepted view today. Chaturvedi (1951) asserts that India is the home of sugarcane, basing his opinion on recorded ancient Hindu mythology, but adducing no other evidence. Crude sugar was being produced by 400 B.C. Brandes (1956) distinguishes three main movements of sugarcane and first of these brought about the introduction of *S. officinarum* to the Solomon islands, New Hebrides and New Caledonia from 8000 B.C. onwards. The second dates from 6000 B.C. and took a westerly direction to Indonesia, Philippines and ultimately to northern India. The third is considered to have occurred in circa A.D. 600 to 1100, reaching various island groups east of the New Hebrides, including Fiji, Tonga, Samoa, the Cook Islands and Hawaii, as well as other parts of Oceania. Then it migrated to Hawaii during 600 A.D. and Prophet Mohammed's army found Sugarcane in Persia during 632 A.D., Sugarcane was commercially exploited in 700 A.D. The first export of sugar from Philippines occurred in 1565 and it has been shown that the general direction of distribution in early times was westward from the Pacific.

The discovery of the Otabeite cane, renamed Bourbon, and its introduction to Mauritius from Tahiti by Bougainville in 1768, exercised a notable influence on the sugar industry of the world. Around 1780, the Bourbon cane was taken from Mauritius to the French West Indies, and introduced to St. Vincent in 1793. These events provided the parent stocks from which most of the sugar of New World, and of considerable proportion of the old, was obtained for the next hundred years until this famous variety succumbed to disease. In 1795 one plant of bourbon was obtained from Santo Domingo by the Island Botanist of Jamaica, a further introduction of the same variety was made to that island by Captain Bligh a year later and obtained one plant of Bourbon from Santo Domingo. This cane was the principal kind grown throughout the Caribbean region and the Hawaii until the effects of a rind disease caused by the fungus *Colletotrichum falcatum* became so serious from 1890 onwards that it had to be replaced by resistant varieties, which though inferior in yield enabled sugar industry to survive. The basis of the sugar industry until shortly after the close of the nineteenth century was a considerable number of variants *of S. saccharum L*. of natural occurrence often called native canes. The Bourbon was the principal variety and other ones being Crytalina, Tanna, and Transparent etc all of which were found in different rind colours and were known by different names. These continued to be cultivated for many years afterwards until they were replaced by artificially developed hybrids having special characteristics including resistance to the diseases which attacked the older canes to an increasing extent as the industry expanded, rendering their cultivation on a commercial scale unprofitable, and in some cases impossible. It is said that the first sugar plant in India was established by the French People at Aska in Orissa in 1824. Not much is known about this factory except that it was maintained by Late James Fredrick Vivian Minchin and that it stopped its operation around 1940. However, the first vacuum pan process sugar plant was set up at Saran in Marhowrah in Bihar in 1904. By 1931-32 there were 31 sugar factories in India all of which were in the private sector. The total production of sugar at that time was only about 1.5 lakh tonnes, whereas the consumption was about 12 lakh tonnes. To meet the domestic demand of sugar, India had to import sugar mainly from Java (Indonesia).

1.e. Botany

All varieties of Sugarcane are species or hybrids of genus Saccharum (Gramineae; Andrpogoneae). Sugarcane is a tall perennial tropical grass (C4 plant) that tillers at the base to produce unbranched stems, 3-4 m or more tall, and about 5 cm in diameter. The basic structure of the sugarcane is closely related to that of other members of the order Gramineae, of which it is a giant member.

The solid unbranched stem, roughly circular or oval in cross section, is clearly differentiated into joints, each comprising a node and an internode. Generally the nodes are placed at an interval of 15 -25 cm, but are much closer at the top of the stem, where elongation is taking place, than at the bottom, where they form part of the rootstock and are essential to the formation of tillers. As a rule, types of canes are preferred which have comparatively long joints and straight stalks, combined with other favorable commercial characters. In commercial production, sugar cane 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 vary in length (5-25 cm), girth (1.5-6 cm in diameter), shape (cylindrical, barrel or bobbin and circular or oval in cross-section), colour (yellow, green, red, purple, black, striped, variegated) and hardness according to the variety and growing conditions.

The leaves of sugarcane are attached to the stem at the bases, alternately in two rows on opposite side of the stem. Each leaf consists of two parts - a sheath and a blade. The leaf has a strong midrib, white and concave on the upper surface, convex and green below.

Two types of root system develop shortly after a sett has been planted: those from primordial of the cutting, which are thin and branched; and those from the primordial of the tillers that are thick, fleshy and much less branched. At first the newly planted seed piece depends entirely on its own roots for the uptake of water and nutrients. Later this function is taken over by the tillers, and sett roots die. Each shoot produces its own root system.

Most *Saccharum* varieties will not flower on day lengths longer than about twelve hours, nor if light is given in the middle of dark period. Generally twelve and a half hours day light and 20-25^{0}C night temperature induces floral initiation if enough inductive cycles are given, probably at least ten. The inflorescence, which is known as arrow, emerges above the mass of foliage, presumably an adaptation to wind pollination. The ultimate branches bear the spikelets, one of which is sessile and other one a stiff pedicel. Both spikelets have two florets, the lower one of which is sterile and represented by a rare delicate pointed lemma or third glume that is shorter than the glumes. Natural pollination is by wind. After pollination, it takes 21–25 days for the seed to fill and mature. The seed is dry one, seeded fruit or caryopsis formed from single carpel, ovary wall (pericarp) being united with seed coat (testa). The seeds are shed within the spikelet, individual florets breaking off at the nodes.

1.f. Importance of Sugarcane to India

In our country, agriculture is not an agri-business, but way of life . Sugarcane, an agro-industrial crop, is an important integral component of the agriculture. It assumes the important position in the economy by contributing nearly 1.9% of National GDP. Sugarcane is cultivated in over 4 million hectares spread over a wide range of agro-ecological situations, both in tropical and sub-tropical regions. The crop sustains second largest organized agro-industry, the sugar industry.

This has enabled us to be the largest producer of sugar and the second largest producer of sugarcane in the world. At present, total production of sugarcane is around 300 million tonnnes. (Shahi. H.N, 2002).

India is the single largest producer of sugar including traditional cane sugar, sweeteners, khandsari and gur equivalent to 26 million tonnes raw value followed by Brazil in the second place at 18.5 million tonnes. Even in respect of white crystal sugar, India has ranked No.1 position in 7 out of last 10 years. Over 11 million tons of refined sugar is produced, accounting for 60% of the total sugar cane cultivated. The total Indian export of the sugarcane in the year 2000-2001 was 811027.5 MT. Punjab is one of the major player in the production of sugarcane in India.

Area under Sugarcane, Yield Per Hectare, Sugarcane Production and Utilisation (India)

Year	Area under cultivation (million hectare)	Yield per hectare	Sugarcane production (million tonnes)	% utilization of sugar	% utilization of gur
1997-98	3.9	66.4	260	49.2	38.8
1998-99	401	68.4	280	56.0	32.0
1999-00	402	70.8	299	59.7	28.4

1.g. Problems of Sugarcane

- **Infestation of major disease - Red Rot**

Red rot, caused by *Glomerella tucumanensis,* (previously known as *Colletotrichum falcatum*), is one of the major constraints in the profitable cultivation of sugarcane. This fungal disease drastically retards the yield and considerably deteriorates the juice quality (Agnihotri, 1990) thus causing considerable loss for both the growers and millers. Many wonder varieties (e.g. CoJ 64) have now gone out of cultivation due to serious infestation of red rot. The symptoms of the disease first appear when the crop is about six months old. At the initial stage, drying of top leaves can be seen. The leaf starts withering and drooping almost all shoots in a clump starts drying one by one. Red lesions with straw coloured centres develop on the midrib of leaves.

Control

Use of disease free or heat-treated setts (MHAT) or resistant variety for planting is recommended. After harvest, collect and burn the plant debris. Grow resistant varieties/use resistant parents in breeding programmes. Dip the setts in systemic fungicide Benomyl (0.1%) solution for at least 30 minutes before planting.

Follow crop rotation.

OTHER PESTS/DISEASES

Apart from the red rot, there are some other pests that are of major concern to the farmers. Among them the prominent one are Top borer, Internode borer, Whip smut, Grassy shoot disease and early shoot-borer. Termites are also contributing to the loss of sugarcane in India. Many states in India are facing severe disease outbreaks and over the years the pathogen have gained virulence and most of the cultivators grown in the field become susceptible (Viswanathan *et al* 1997). It is also observed that due to the development of the new variants of pathogens, the newly released varities succumbed to pathogen (Padmanabhan et. al. 1996)

- **NON AVAILABILITY OF HEALTHY SEED CANES**

Healthy seed cane supply is the lifeline in the productivity of the sugarcane. Since the disease is carried to the planting material and sugarcane, it is very important to maintain the healthy cane supply system for improving /sustaining the productivity. Therefore it is imperative to adopt utmost care for the use of only healthy seeds as planting material. For this, three-tier production programme i.e. breeder-foundation-certified has to be followed which may be costly. Moist hot air treatment (MHAT) treatment can be given to the seed material and this treatment can only be given at the breeder seed stage only but not at the commercial level. The area under sugarcane has reduced due to non-availability of superior varieties. In 2000-2001, sugarcane area in the Punjab fell down to 145,000 ha. Primary reason for this drastic reduction was infestation of diseases, mismanagement of sugar mills and untimely payments to the farmers in the preceding year. These non availability of healthy seed cane and infestation of diseases call for an integrated approach for solving the problem.

- **MINIMUM SUPPORT PRICE**

A higher MSP promotes sugarcane as a diversification crop and encourage farmers to break free from the wheat-paddy cycle. While the MSP for sugarcane fixed by the Centre for 2001-02 is Rs 620.50/tonne, the growers are paid higher due to the State Governments announcing advisory prices (SAP). Uttar Pradesh' s SAP ranges from Rs 980 to Rs 1,000 a tonne based on recovery, while in Haryana, it rules at Rs 750-1,100. In the southern States, it is between Rs 750 and Rs 780. However, despite the directives from the Central and State Govt to provide farmers MSP and arrears, mill owners refused to observe the rule. And due to this situation, even in this year 2003, farmers face huge losses to sell canes at a lower ate. But there are other hurdles, which discourage farmers from taking 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 a recent press report, sugar mills in Haryana owed Rs 100 crore to growers while the arrears in UP amounted to a staggering Rs 1,000 crore. The situation countrywide is so grim that the payment of sugarcane arrears to farmers was included in the Govt of India's recent package for the drought-hit. Since the income generated by sugarcane is only once a year, there is a dire need to diversify the cropping system by introducing intercropping to generate mid-season income for the farmers and additionally fulfilling the household requirement of food, besides mitigating the ill effects of sugarcane monoculture.

1.h. Integration of Tissue Culture with Sugarcane

Plant regeneration from tissue culture of sugarcane has been successfully applied to breeding programs for rapid screening of clones for disease resistance, salt tolerance, drought tolerance, herbicide resistance and early maturity and high sugar. A newly identified variety with desirable character, e.g pest/disease resistant, high sugar content, stress resistant can be propagated through tissue culture and made available to the farmer for commercial benefit that is otherwise not possible through conventional means that take appox 8-10 years. Yield losses due to diseases could be reduced by using resistant varieties. (Krishnamurthy and Tlaskal 1974) obtained disease resistance sub clones through tissue culture. Liu *et al* (1983) developed a calli clone containing an average of 2.35% more sucrose than its donor and had taller and thicker stalks. In vitro selection from callus culture whereby the selection pressure is imposed by adding a toxic substance from the environment so that only the mutated cells survive and hence a more resistant form is developed, e.g. for salt tolerance desirable mutants can be isolated from the cells exposed to high salinity. An in vitro selection system for salt stress and eye spot disease resistance has been developed by Maribona *et al* (1986). This system is able to discriminate pathological and salt stress adaptation for genetic mutation. However, it is also imperative to check the persistence level over generations and only stable mutants showing desirable characters can be released. Development of molecular markers for disease resistance, high sucrose and other characters are been undertaken in research organizations (e.g, Sugarcane Breeding Institute, Coimbatore) and the benefits of these technology can only be translated when improved varieties will be released for the benefit of the farmer. Tissue culture technology can play a major role for efficient dissemination of these. An integrated approach including a research organization for identifying and selection of a new / important variety, mass propagation and Sugarcane industry support in raising a mother nursery for bulking up has been in need of the hour.

2.0 Review of Literature

Tissue culture is the cloning of plants from mother plant. Tissue culture materials are uniform at maturity and this makes it easy for harvesting. Plant tissue culture is a versatile cloning technique offering benefit of scale, scope and uniformity. It requires sophisticated equipments, skilled manpower, dedicated research and is labour intensive. Tissue culture technology can ensure virus-free, disease indexed and high yielding planting material, which will help to increase yield, productivity, uniformity of produce, reduced harvesting time and reduced wastage.

Several of the authors and scientific institutes all over the world are working on various aspects of Sugarcane ranging from Agronomy, Plant protection, Crop improvement , mass propagation, genetic transformation etc. We have referred some of the important works pertaining to standardizing of tissue / callus culture of Sugarcane.

Plant regeneration from shoot tip culture of sugarcane using Murashige and Skoog (MS) medium supplemented with 0.2 mg/lt BA and 0.02 mg NAA/lt have been reported. Shootlets were multiplied 4-fold every 2 weeks by sub culturing in the same medium. Further multiplication was carried out on solid MS medium containing the same growth regulators, before enhancing shoot growth on medium containing 1 mg 2,4-D/lt and 15% (v/v) coconut water, and finally stimulating rooting on MS medium with 1 mg IBA/litre. (Naritoom, K. *et al.*, 1993). However, it is later developed that in Sugarcane, once axillary bud proliferation has been started, liquid culture medium can be used for further multiplication. This will reduce usage of gelling agent like Agar, which is very costly, and add considerably towards the cost of development of plantlets. The use of different gelling agents (agar and agarose) and support materials (filter paper bridge, cotton cloth bridge and adsorbent cotton) as well as shaken and static liquid (control) cultures was studied in order to improve in vitro shoot multiplication and vigour in sugarcane (Lal, N. *et al*, 1993). Sterilized 2-3 mm shoot tips of sugarcane were cultured on modified Murashige and Skoog medium containing 3 or 4 mg BAP and 0-2 mg IAA/lt. Percentage induction of multiple shoots, and mean multiple shoot number, were highest (90% and 16.5, respectively) with 3 mg BAP + 1 mg IAA/lt. Roots were induced on half-strength MS medium containing 2 mg IBA + 1 mg IAA/lt (Dhumale, D. B. *et al.* , 1994).

Rapid multiplication of commercial sugarcane varieties through tissue culture was demonstrated in MS medium supplemented with 0.5 mg IAA, BAP and Kinetin (70% shoot regeneration) and rapid shoot multiplication was achieved by sub culturing the established shoot clumps in MS medium supplemented with 0.1 mg/lt IAA, 2.0 mg/lt BAP, and 1.0 mg/lt Kinetin. Rooting (85-92%) was induced by transferring shoot clumps on 1/2 MS medium containing 2 mg/lt NAA and 1.0 mg/lt IBA. (Pawar, S. *et al*, 2002).

Effect of growth regulators on in vitro multiplication of sugarcane cultivars (Co-86032, Co-740 and Co-8014) were used to study the effect of different levels of kinetin, benzyl-aminopurine (BAP) and coconut water(CW) on shoot multiplication, length of main shoot and number of leaves on main shoot. MS medium was supplemented with 3 levels of kinetin (0.5, 1.0 and 1.5 mg/lt), 4 levels of BAP (0.5, 1.0, 1.5 and 2.0 mg/lt) and 2 levels of CW (10 and 20%). The highest shoot multiplication ratio for all cultivars was recorded in the MS medium supplemented with 1.5 mg kinetin/lt, 1.5 or 2.0 mg BAP/lt and 20% CW. The treatment 1.5 mg kinetin/lt+1.0 mg BAP/lt+20% CW gave the highest values for length of main shoot in all cultivars, invitro plantlets treated with 1.0 mg kinetin/lt+1.0 mg BAP/lt+20% CW gave the highest number of leaves on the main shoot in all cultivars. (Pawar, S. V. *et al.*, 2002).

Another interesting thing was observed when the effect of explant source and genotype on growth of sugar cane in vitro was observed. 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 B 6504 and M 554/79, 8.3% and 12.5% of axillary buds, respectively, formed shoots. On the other hand, 26.3% and 36.4% of apical buds developed into shoots. Best response was obtained with variety M 261/78, with more than 66%

of explants developing into shoots. M 52/78 responded poorly (Mulleegadoo, K. D. and Dookun, A. 1999).

An efficient protocol for micro propagation of sugarcane using shoot tip explants was developed in which sugarcane cultivars inoculated on MS agar-gelled medium containing benzyladenine, kinetin and gibberellic acid (0.5 mg/lt each) + sucrose (30 g/lt) at pH 5.8. Clumps with well-grown shoots were transferred on 1/2 strength MS liquid rooting medium supplemented with NAA (5.0 mg/lt) and elevated sucrose level (60 g/lt) for induction of rooting. 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 (Singh, B., *et al*, 2001). It was reported that the 4-mm size of meristem tips was the most suitable for establishment of culture. When meristem tips were treated with solution of ascorbic acid (100 mg/lt) + citric acid (150 mg/lt) for 10-15 minutes, phenolics could be controlled (Kazim Ali *et al.*, 2001).

The highest mean rate of callus induction was produced on MS medium + 3% sucrose + 3 mg/lt 2,4-D + 0.5 mg benzyladenine/litre. On this medium, callus induction from spindle explants was greatest in the genotypes CoS 767, CoJ 64 and S 2536-82 (Cheema, A.S., *et al*, 1992). Sterilized explants of sugarcane variety Co 740 were cultured on semisolid MS medium containing 100 mg myo - inositol and 3 mg 2,4-D/lt and 10% v/v coconut milk, and incubated (Mohatkar, L.C., 1993) for callus induction.

An interesting observation involving immature inflorescence segments of sugarcane (Saccharum spp.) breeding lines (87-588, 87-693 and 87-696) were cultured on 4 versions of MS based media containing various concentrations and combinations of 2,4-D, kinetin, BAP [benzyladenine], casein hydrolysate and calcium pantothenate. Calluses could be initiated from all parts of the inflorescence segments. In terms of callusing frequency, the most important factor was the age of the explants. As long as it was at the period of pollen mother cell to tetrad, all of the 3 genotypes were capable of producing a large quantity of callus. Callusing frequency was significantly higher following cold (13 deg C) storage for 2 or 3 d than following natural night temperature treatment (Liu, M.C., 1993).

Calluses were derived from the innermost, unfurled, leaf spindles of sugarcane cv. CoJ-76 and cultured on MS medium, supplemented with 3 mg/lt 2,4-D and 0.2 mg/lt benzylaminopurine (BAP) and maintained under dark conditions. Calluses were then separated according to morphological appearance and maintained by regular subculture on MS medium supplemented with 2 mg/litre 2,4-D and 0.2 mg/lt BAP at 15 days intervals (Gupta, J. N. *et al* , 1995).

Kharinarain, R. P., 1996, reported that Murashige-Skoog (MS) medium supplemented with 3 mg 2,4-D/lt and 5 mg diethyldithiocarbamate/lt was best for the formation of morphogenetic calluses.

Segments from young leaf bases of 4 clones were cultured on MS medium supplemented with 10% coconut milk and 0.5-3.0 mg 2,4-D/lt. Callus formed within 7-10 days and somatic embryos were obtained within 15-20 days of callus initiation(R. Islam *et al*, 1996). Various factors affecting efficient plant regeneration from long-term maintained callus cultures were investigated in three sugarcane cultivars, viz. CoJ 64, CoJ 83, and CoJ 86. Murashige and Skoog (MS)+2,4-D (4 mg/lt)+BAP (0.5 mg/lt) induced excellent callusing in the spindle explants of all the three cultivars (Ajinder Kaur *et al*, 2001). The effects of 2,4-D (2, 3 and 4 mg/l), alone or in combination with benzyladenine (0.5 and 1.0 mg/l), on the somatic embryogenesis of sugarcane cv. Co Si 95071 were investigated. Growing tips collected from 4-month-old plants were placed in Murashige and Skoog medium and incubated at conditions of 24-26 deg C, 5.8 pH, 16 h light and 8 h darkness. After 3 weeks of incubation, non-embryonic calluses, and cream-coloured, compact and nodular somatic embryos, appeared on cut edges of leaf bits. More somatic embryos were formed using 2,4-D combined with benzyladenine. The treatment with 3 mg 2,4-D + 0.5 mg benzyladenine produced the highest number of somatic embryos (Geetha, S. ; Padmanabhan, D., 2001).

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3.0 Material and Methods

3.a. Collection of Germplasm

We collected the plant material from Ladowal seed farm, Punjab Agricultural University (PAU), Ludhiana from the Department of Plant Breeding. The explants from 6-8 month old, healthy, disease free seed canes of variety CoJ 85 were collected.

3.b. Medium

The appropriate composition of the medium largely determines the success of cultures. Plant material does vary in their nutritional requirements and therefore it is often necessary to modify the medium to suit a particular tissue. The basal medium employed for the culture of sugarcane is MS medium (Murashige and Skoog 1962). A variety of growth regulators such as 6- Benzyl amino purine (BAP), alpha-Naphthalene acetic acid (NAA), 3-Indole Butyric acid(IBA) and 2,4-dichlorophenoxy acetic acid (2,4-D) were added to the medium singly or in combinations at various concentrations and were used for initiating different experiments. The concentrated stock solutions of the major salts, minor salts and vitamins are prepared to be used in the preparation of the media and stored under refrigeration. Auxins were dissolved in 1N KOH and cytokinins in 1N HCL before making up the final volume with distilled water. Iron EDTA stock solution was stored in amber coloured bottle.

- The medium was prepared by adding appropriate quantities of the stock solutions and correct volume was made up with the distilled water. The pH was adjusted in all cases to 5.8 by using 1 N KOH and 1 N HCL and agar 0.8%(w/v) were used for semi solid medium for culture initiation/establishment only and liquid medium were used for multiplication and rooting of microshoots. Before autoclaving, the media was poured into washed and dried test tubes (upto 20ml) and or culture bottles (15-20ml for liquid culture) which are then, capped and labelled properly. These were then autoclaved at 121^0C for 15 minutes at 15-psi pressure and transferred to the inoculation room where they were stored under aseptic conditions till their use.

Composition of Murashige and Skoog (1962) Medium

Constituents	Amounts (mg/l)
A. Macronutrients	
NH4NO$_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
B. Micronutrients	
H_3BO_3	6.2
$NaEDTA.2H_2O$	37.30
$MnSO_4.4H_2O$	16.9
$FeSO_4.7H_2O$	27.8
$ZnSO_4.7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.7H_2O$	0.025
C. Vitamins	
MYOINOSITOL	100
GLYCINE	2.0
NICOTINIC ACID	0.5
PYRIDOXINE HCL	0.5
THIAMINE HCL	1.0
SUGAR	30000
AGAR	6000

D. Growth regulators (when required) SIGMA

alpha-Naphthalene acetic acid (NAA)

Indole acetic acid (IAA)

2,4- dichlorophenoxy acetic acid (2,4-D)

6- Benzyl amino purine (BAP)

Kinetin (Kn)

Choice of Explant

The tissue taken from a plant or seed and transferred to a culture medium to establish a tissue cultures system or regenerates a plant. The choice of the explant depends upon the methods of the shoot multiplication to be followed. All plant organs viz. nodal segments, internodal segments, shoot tip, root, cotyledons, epicotyl, hypocotyl, leaf, petiole, anthers and ovule etc are known to give rise to complete plants.

For micro propagation, where aim is to get identical plants, it is advisable to initiate cultures from explant from preexisting meristems. It is necessary to know the origin (variety, cultivar). In adult plant to such explants exists i.e. shoot tip and nodal explant (stem portion to which leaf is attached). For most micro propagation work the explant of choice is an apical or an axillary bud. Usually the explant are more responsive to culture treatments if they are collected during the period of active growth.

3.c. Explant Sterilization

- Select the disease free and young, healthy sugarcane tops, as their cells will be more likely to have retained their totipotency. Remove the young leaves, from the top portion of plant and excise the spindle from the top.
- The collected explants are partially trimmed off and then washed thoroughly under running tap water for 10 minutes each for three cycles to wash off external dust/contaminant.
- After this, spindles were again washed with a liquid detergent (Rankleen, Ranbaxy, India) adding a few drops of Tween-20 for 15 minutes. This treatment should be done twice to avoid any chance of contamination. The

detergent acts as a wetting agent and allows the entire surface of the spindle to be exposed for anti microbial agent.

- After these treatments, explants were washed again in sterile water for 10 minutes.
- After these treatments, explants were washed again under running tap water for 10 minutes.
- After these initial washings, the explants were kept in an aqueous solution of Bavistin (BASF India Limited) [1% w/v] for 15 minutes.
- The explants were firstly taken out from the Bavistin solution using forceps and transferred to sterile conical flask, which were then washed thoroughly in sterile distilled water (4 washings).
- After this treatment, the explants were taken inside the laminar hood for further sterilization. Inside the laminar hood, sterilization with 0.1% mercuric chloride is done for approximately 8 minutes. When the explant has been "surface sterilized", it is removed from the sterilizing solution and rinsed three times in sterile, autoclaved distilled water to completely remove any remaining mercuric chloride.
- These last steps are performed inside a laminar flow hood to maintain the axenic condition of the explant and to prevent the re-introduction of contaminating microbes. Finally all the extra water is removed from the spindle and the explant is then ready to be trimmed if necessary.

3.d. Initiation and Culturing of the Spindle

- Sterilized explants were transferred aseptically from the conical flasks to sterile glass plates under laminar hood in inoculation chamber for making them into the suitable sizes (3.5 – 4.0cm). All the outer whorls of the spindle should be removed with a sharp blade attached to scalpel and the precaution to be taken so that spindle should not get injured or broken.
- Then, a fresh cut to be given on both basal as well as the top portion of the spindle. Again, rinse the forceps in the 70% ethanol and flame the forcep and

allow it to cool. Remove the lid from one test tube and test tube mouth need to be flamed to avoid further chances of contamination.

- Place the spindle in the test tube with the long forceps without touching the rim of the test tube and place no more than one section in each test tube. Carefully replace the lid onto the test tube, lightly flame it and then and seal with parafilm.

Return the forceps to the ethanol and mark the variety of the sugarcane on the test tube along with the date. Repeat the same procedure for all the spindles available and finally put test tubes in the rack and place them in growth room. Maintain 25±2^{0}C , with 16 hours daylight and 8 hrs night break.((2000-3000 Lux).

3.e. Establishment of the Cultures

After two cycles of transfer of the spindles to the solid medium, the spindles had established themselves and they turned green in colour from yellowish green. Then spindles were inoculated into the liquid medium, which had the same composition as that of the initiation media excluding the agar. With a clean forcep, the spindles were taken out of the test tubes, medium adhered to the basal portion of the spindle was removed, undesirable / brownish leaves / portions were removed and well-established cultures with 1-2 shoot buds were transferred to the culture bottles containing autoclaved liquid medium (15-20 ml per bottle). Then the bottles were labeled and placed in the culture room under the standard conditions of temperature (25± 2^{0}C) for 16 hours of daybreak (2000-3000 Lux).

3.f. Multiplication of the Shoot by Repeated Subcultures on Multiplication Medium

Concept of Multiplication

The multiplication of the explant is a crucial stage in the propagation of any species for commercial exploitation and the most rapid rates are required. The most common additives to standard media are cytokinins usually as BAP or

Kinetin. Typically the same medium and environment conditions are used for both stage I and II.

1) Enhanced Axillary Branching

The axillary bud present in the axil of the each leaf either develops into a single shoot or forms a cluster of shoots. The presence of cytokinin influences the rapid emergence of axillary buds by reducing apical dominance.

2) Adventitious Bud Formation

Buds arising from any part other than the leaf axil or shoot apex are called adventitious bud. In nature also, a number of plant species produce adventitious buds from different organs e.g. leaf in Begonia, root suckers in Sheeham etc. Infact propagation through adventitious bud is a standard horticulture practice. This method can't be applied for chimeric plants (plants with variegated leaves) as it results in solid lines.

3) Through Callusing

Plant cells are totipotent i.e. able to multiply indefinitely later differentiate to form the whole plant. In tissue culture the differentiated cells first differentiate and give rise to the mass of cells commonly known as callus. The callus either give rise to shoot bud or the bipolar structure resembling embryo. These are known as somatic embryo. There are certain doubts raised on the clonal uniformity of the plants produced. Since the division in the callus cells are very fast, the chromosome number might vary and different type of abnormalities might occur thus plants generated from callus source may not be identical to the mother plant. This method however is of the immense importance when the aim is to induce variability especially in self-pollinating species with narrow genetic base.

4) Subculturing

This susceptibility to culture components and as a consequence, the reaction of the explant, can change within culture and number of subculture. On the same medium, cultures, originally yielding axillary shoots, can produce abundant adventitious shoots after number of subcultures. Generally 10 –12 subcultures is considered to be maximum.

Protocol For Multiplication

- Repeat the preparation and sterilization steps for the medium, instruments and chamber as before. Sterilize hands as before too. Transfer the containers of shoots to one side of the cabinet and the sterilized medium at the other side.
- Transfer several shoots from the culture bottle to a sterile glass plate using flamed sterilized scalpel. Remove the brownish coloured leaves from the clump and sub divide the clump into the smaller clumps.
- Transfer the mass or the shoot into a new culture flask containing multiplication medium (15-20ml) for which different combinations have been made. All this work was done with extreme care and inside the laminar hood to avoid any possible chance of contamination.
- Incubate the flask in the culture room under light and repeat the step 2-4 times every 2-3 weeks so that a mass of shoots is formed. As a general observation, sugarcane shoots can multiply 2.5 – 3 times initially every 2-3 weeks.
- If a sterile environment was not maintained, contamination will be obvious within 3-4 days.

DIFFERENT COMBINATIONS OF THE MULTIPLICATION MEDIUM

(with medium code: C1-C16)

GROWTH REGULATORS (mg/ltr)	0.2 BAP	0.5 BAP	1.0 BAP	1.5 BAP
0.2 Kn	C 1	C2	C3	C4
0.5 Kn	C5	C6	C7	C8
1.0 Kn	C9	C10	C11	C12
1.5 Kn	C13	C14	C15	C16

Control: C0 – MS Basal medium

3.g. ROOTING OF PLANTLETS

CONCEPT OF ROOTING

Once the sufficient number of shoots have been generated, portion of explant that contains one or more than one shoots could be transferred to a medium that contains higher concentration of auxin, resulting in root formation. The production of the roots is easily achieved in some species by reducing the cytokinin level or in MS Basal medium with or without the addition of extra root promoting auxins, especially IBA (0.2-0.5mg/l). A lower concentration is often require e.g. Knop's medium, half strength MS. Activated charcoal added in the medium eliminate the residual effect of cytokinin by adsorption. Phenolic co-factor often enhances the rooting temperature. Rooting is initiated in stage III.

Protocol For Rooting

- In the laminar flow, under sterile conditions, remove the para-film and cap from the culture bottle and use the forceps to carefully remove the explant from the multiplication medium.
- Place the multiplied shoot mass on a sterile petri dish or on the sterile glass plate. Using a sterile scalpel carefully remove or cut plantlets away from the mass of shoots/clumps. Remove the undesirable portion of the explant and using sterile forceps rinsed in 70% ethanol and flamed, carefully place plantlets into the rooting medium.
- Note that lower side of the shoot remains in contact with the media and shoot remains straight. Carefully cap the culture bottle, label them properly and return them to the rack for 2 - 4 weeks under the same condition. During this time, the shoots will continue to grow, however, most of the plant's energy will be focused into producing roots.

DIFFERENT COMBINATIONS OF THE MEDIUM FOR ROOTING(with Medium code: (RT 1- RT10)

SUGAR 30gm/L	IBA 0.5ppm	IBA 1.0ppm	NAA 2.0ppm	NAA 5.0ppm
FULL MS	RT 1	RT 2	RT 3	RT 4
HALF MS	RT 5	RT 6	RT 7	RT 8

SUGAR 70gm/L	NAA 5.0 ppm	NAA 3ppm + IBA 2ppm
FULL MS	RT 9	RT 10

Control: RT0 (MS basal)

3.h. Transplantation of Plantlets

Concept for Transplantation

This is the final stage and required careful hardening of plants .The transition of plantlets from completely controlled conditions (heterotrophic growth) to outside environment (autotrophic) should be gradual in order to prepare the tissue culture raised plants to survive in the field condition. This is called acclimatization. The plants produced in tissue culture, although green in color do not prepare sufficient food for their survival. Also inside the culture vessel the humidity is high and thus the natural protective waxy covering of cuticle is not fully developed. In few species, stomata do not close properly, therefore immediately after transfer plants are maintained under high humidity (above 90%) and it is gradually reduced over a period of 6 to 8 weeks. During this time the plants attain ability to synthesize more food and develop cuticular wax covering. Plants are later transferred to larger container with a compost based mixture. Plants are maintained under shade and once they have started producing and maintaining newer leaves/roots, they are the ready to be transfered in open nursery. Tissue culture propagated plants should be retained in the nursery for the same duration as the conventionally raised plants.

Protocol For Transplantation

- After 15-20 days of culture on modified MS media meant for rooting, the sufficiently rooted plantlings were transplanted for hardening prior to their final transfer to the soil. The rooted plantlets were taken out of the culture bottles using forceps with extreme care to avoid any mechanical damage to the plantlets and root system. These were then thoroughly washed in tap water or lukewarm water to remove any remaining medium to avoid any future infection of plantlet. Again, plantlets were dipped in Bavistin(1gm/lt) solution for appox 2-3 min.
- After this, the plants are carefully planted in the polybags containing agro peat mixed soil (cocunut peat compost, supplied by M/s Varsha Enterprises, Bangalore, India)by inserting a hole in the middle of the potting mix and gently insert the roots in that hole. It is to be taken care that the roots at the time of transplanting should not be too long so that they bent during transplantation.
- These are then thoroughly watered and kept in poly house under humidity range of >75% -80% for about 2-3 weeks, with. The poly house is covered with agro net on the top and the humidity is maintained with intermittent misting. After this period in poly house, these plantlets are generally transferred to shade house in which they should be kept under the humidity range of 60-70%. Watering is done as per requirement.
- When the roots are well established and the plants are acclimatized (this should take about 2 weeks), they can be given light fertilizer and be treated like any other plant. It is advisable to gradually increase the light intensity and transfer the plants to open nursery for final hardening.

4. Regeneration of plants through Callus formation

Concept of Callus

A mass of unorganized cells resulting either as a consequence of wounding in plants or in tissue culture.(Mascarenhas, A.F. 1997). The technique of callus (tissue) culture was first developed in the late 1920s and 1930s and was one of the primary methods of tissue culture for many years. Callus cultures have been frequently used in physiological, biochemical, and genetic experiments to unfold the mystery of hitherto unexplained plant processes.

Unlike animals, where differentiation is generally irreversible, in plants even highly mature and differentiated cells retain the ability to regress to a meristematic state as long as they have an intact membrane system and a viable nucleus. When non dividing, quiescent cells from differentiated tissues are grown on a nutrient medium that supports their proliferation. The cells first undergo certain changes to achieve meristematic state these include replacement of non-functional cellular components damaged by lysosomal activity during the processes of cytoquiescence. The phenomenon of a mature cell reverting to a the meristematic state and forming undifferentiated mass known as callus is termed as dedifferentiation. Multicellular explant generally comprise of cells of diverse type. As a result the callus derived from it would be heterogeneous with respect to the ability of its component cells to form a whole plant or plant organs and the phenomenon is known as redifferentiation and the process of induction of roots and shoots in callus cultures is referred as organogenesis.

For plant cells to develop into a callus the nutrient medium should contain plant hormone essentially an auxin, a cytokinin and a gibberellin. The absolute amounts of these, which are required, vary for different tissue explants from different parts of the same plant and for the same explant from different genera of plants.. Most of media in common use consist of inorganic salts. Trace metals, vitamins, organic nitrogen sources (glycine), inositol, and sucrose and growth regulators. Relatively high ratio of auxin to cytokinin is required for root induction and high ratio of cytokinin to auxin is required for shoot induction.

When subcultured regularly on nutrient medium callus culture will exhibit an s–shaped or sigmoidal pattern of growth. There are five phases of callus growth, these phases are: -

- A lag phase, where cells prepare to divide
- An exponential phase, where rate of cell division is highest
- A linear phase, where cell division slows but the rate of cells expansion increases

¬ A deceleration phase, where the rate of cell division and elongation decreases

¬ A stationary phase, where the number and size of cells remain constant.

Callus growth can be monitored by fresh weight measurements, which are convenient for observing the growth of cultures over time in a non-destructive manner. Dry weight measurements are more accurate the fresh weight, but this method requires sacrifice of the sample.

Factors Affecting Callus Induction

- Position of the explant on the plant as well as size of explant.
- Genotype of the explant.
- Physiological state of the donor plant and explant.
- Composition of culture medium.
- Environment under which cultures are grown i.e. light, temperature, and humidity.

Callus cultures can be induced to undergo an entirely different development process under certain nutritional and hormonal conditions, which is known as somatic embryogenesis. In this process the callus cells undergo a pattern of differentiation similar to that observed in zygotes after fertilization and produce embryoids. Such somatic embryoids differ from normal embryos in being produced from somatic cells instead of fusion of two germ cells.

Uses of Callus Cultures

Callus cultures are useful for many purposes of pure and applied research among these are:

- Their use for the synthesis of starting compounds that are subsequently modified to yield the desired product.
- Their use as starting material for the vegetative propagation of plants

- Their use as basic material fore high-yield cultivars (maintenance breeding).
- Their reverting to tissue cultures allows the conservation of virus- or fungi-free and resistant cell lineages

CALLUS INITIATION

Explant Sterilization

Select the disease free and young sugarcane tops and remove the young leaves, from the top portion of plant and excise the spindle from the top. The collected explants are taken and are partially trimmed off and then washed thoroughly under running tap water for 30 minutes. After this they were again washed with a liquid detergent (Rankleen, Ranbaxy, India) and then immersed in water containing few drops of Tween-20 for 20 minutes This treatment should be done twice to avoid any chance of contamination. The detergent acts as a wetting agent and allows the entire surface of the spindle to be exposed for anti microbial agent. After these treatments, explants were washed again under sterile water for 10 minutes. After these initial washings, the explant were kept in an aqueous solution of Bavistin (BASF India Limited) [1% w/v] for 15 minutes. The explants were firstly taken out from the Bavistin solution using forceps and transferred to sterile conical flask which were then washed thoroughly in sterile distilled water (4 washings). Sterilization with 0.1% mercuric chloride is done for approximately 7-8 minutes in the laminar flow. When the explant has been "surface sterilized", it is usually removed from the sterilizing solution and rinsed three times in sterile, autoclaved distilled water to completely remove any remaining mercuric chloride. These last steps are performed inside a laminar flow hood to maintain the axenic condition of the explant and to prevent the re-introduction of contaminating microbes.

Pretreatment of Coconut Water for Culture Medium

Firstly the coconut water is removed from the green coconut and collected inside the clean beaker and then it is filtered with the help of cotton and filter paper. Now the filtrate is taken in the flask and placed in the microwave for 5-7 minutes and now again it is filtered. It is allowed to cool down and ready for use.

Initiation of the Explant for the Callus

Leaf explants were inoculated directly into the medium (2-3 per bottle) and the spindle was inoculated at a rate of one segment of spindle per bottle. The medium employed was MS Basal with different concentration and combinations of phytohormones such as 2,4-D. After inoculation the culture bottles were properly capped and sealed. With sufficient labeling these are transferred to the incubation room where they are incubated 25± 2^0C in the dark room or the culture bottles are incubated in the black paper.

5. DNA ISOLATION:

To find out any possible somaclonal variation in plants regenerated through micro propagation and for genetic analysis of plants growing in the natural conditions and plants raised through micro propagation we have to isolate DNA of both the plants. For the isolation of DNA we used the CTAB Precipitation method of genomic DNA isolation. The protocol CTAB (Cetyl trimethly ammonium bromide) DNA isolation was initially used in bacteria (Johns, 1953) and later modified to obtain DAN from plants (Murray & Thompson 1980). For the isolation DNA of both the samples we use CTAB plant extraction kit provided by GENEI, Bangalore. The protocol and solutions used in the kit are as fallows.

Solutions of CTAB DNA isolation kit

Solution A (CTAB extraction buffer)

CTAB	- 2% (w/v)
Tris-HCl	- 10 mM (pH 8.0)

EDTA - 20 mM (pH 8.0)

NaCl - 1.4 M

Solution B (Extraction buffer)

Tris-HCl - 100 mM (pH 8.0)

EDTA - 100 mM (pH 8.0)

NaCl - 250 mM

Solution C (CTAB Precipitation solution)

CTAB - 1% (w/v)

Tris-HCl - 50 mM (pH 8.0)

EDTA - 10 mM (pH 8.0)

Solution D (High salt TE buffer)

Tris-HCl - 10 mM (pH 8.0)

EDTA - 0.1 mM (pH 8.0)

NaCl - 1.0 M

Procedure

The young and soft plant tissue (~1.2 g) was pulverized to a fine powder in the presence of liquid nitrogen and was distributed equally to three microfuge tubes (2.0 ml capacity). Immediately 0.9 ml of solution A + 2% (v/v) â-mercaptoethanol (at 65º C) was mixed to the tissue to wet it thoroughly. The tubes were incubated at 65• C for 1 hr. with occasional mixing, after every 15 min. Equal volume of chloroform: iso-amyl alcohol (24:1) was added to it. The solution was mixed well by inversion for 7 min. and then centrifuged at 8000 rpm for 5 minutes. The upper aqueous layer was recovered, to which, 1/10th volume (100 ì l) of 65• C solution B was added and this solution was mixed well by inversion for 5 min. This solution was extracted in the same way with equal volume of chloroform: iso-amyl alcohol (24:1) and the upper aqueous layer was recovered. To this solution, 1 volume (900 ì l) of solution C was added. The solution was mixed well and was incubated at 65• C for ½ hr. This solution was centrifuged for 5 min. at 5000 rpm. The pellets were resuspended in ~500 ì l of solution D by intermittent incubation at 65• C. 300 ì l of isopropanol was added to each, mixed well and this was kept at 4• C for 15 min. This solution was centrifuged at full speed for 15 min., when the pellets were washed with 80% ethanol. The pellets were air-dried and were finally resuspended in 60 ì l of TE buffer and store at –20°C.

6. RESULTS AND DISCUSSION

6.a. Initiation and Establishment

After the inoculation of the spindle(s), it was found that the sterilization protocol followed for the sterilization of the explants was reliable, as out of 12 explants, only two had reported the contamination so the survival rate comes out to be 83.33%.

Table 1

Observation of initiation:

Number of explants taken	Contamination reported	Survival percentage
12	2	83.33%

After the incubation of the spindles in the controlled conditions of temperature and humidity, it was observed that the spindle had changed its colour from pale yellow to green in colour in the first week but during this period there was not any significant growth of the spindle. It was also found that the medium at the base of the spindle turn brown which is assumed to be due to the release of phenolics in the medium after 12-14 days and there was not any significant decrease in the release of the phenolics even though some of the medium combination was supplemented with an anti-oxidant i.e. citric acid (50mg/l). The explants were inoculated again after every 7-9 days into fresh medium (of same composition).

Table 2

Observation of browning:

Medium	Number of Spindles	Observation (+ or – browning of the medium)
SI-3 (0.5 mg/lt BAP + 0.5 Kn mg/l)	**2**	**+**
SI-3 CA (0.5 mg/lt BAP+ 0.5 mg/lt Kn + Citric Acid 50 mg/l)	**2**	**+**

After 14 days of incubation, it was observed that there was increase in the size of the spindle and whole of the spindle had turned green. The spindle had sprouted at the base but not much growth was observed in it. The medium, which had 0.5 mg/l of BAP and 0.5 mg/l of Kinetin (SI-3), had shown the best results of initiation as it shows 75% bud break. Pawar *et al* observed approximately 70% shoot regeneration after 4 weeks of incubation. However, in control, we found only 30 % bud break and also the growth and appearance of the sprouted bud were weak as compared to the mediums using phytohormones. It is therefore observed that phytohormones (cytokinins) have a definite role in axillary bud initiation.

Table 3 Observation of initiation on different media combinations:

Media Combinations	No of explants inoculated	Discard	No of explants showed bud break	% of bud break
MS (control)	**4**	**1**	**1**	**30%**
MS + 1.0 BAP + 1.0 Kn	**4**	**1**	**2**	**66%**
MS + 0.5 BAP + 0.5 Kn	**4**	**0**	**3**	**75%**

After 30 days of incubation the sprouted bud had grown considerably especially in the medium containing MS + 0.5 mg/lt BAP + 0.5 mg/lt Kn. The length and basal growth of the sprouted buds are observed significantly higher than the other medium containing MS + 1.0mg/lt BAP+ 1.0 mg/lt Kn.

DIFFERENT STAGES OF INITIATION OF SPINDLE OF CoJ 85 IN SI-3 MEDIUM

6.b. MULTIPLICATION RESULTS

After 4 weeks of subculturing in the solid medium (3 times with an interval of 7-10 days depending upon the conditions of the spindle), the parent spindle along with the newly formed axillary bud sprouts was transferred to the liquid culture medium having different concentration of the plant growth regulators. These explants were inoculated into the fresh culture bottles having approximately 15-20ml of the medium. We have observed, initially mass of axillary shoot buds appears to be increased with each sub culturing stage upto 2-4 sub culturing in the liquid media. To reduce cytokinin carry over effect, we have inoculated all the cultures into MS Basal media before experimentation with different media combination. Then, we subjected them to multiply under different media combinations and recorded following observation.

Table 4

Observation of different media combinations for multiplication:

Each culture jar: 2 clump each having 6-8 shoots on inoculation day

No of replicates: 3

Media combination	No. of shoots / jar on inoculation	No. of shoots / jar on 14 days	No. of shoots / jar on 21 days
Control, C0	12.81 ± 1.4	21.11 ± 1.7	24.2 ± 2.0
C6	14.33 ± 2.3	44.33 ± 2.2	46.33 ± 2.1
C8	12.33 ± 2.0	37.33 ± 2.0	39 ± 1.7
C13	12.33 ± 1.2	35.67 ± 2.3	36 ± 1.7
C15	16.67 ± 2.5	54.33 ± 1.8	57 ± 2.4

In this case, we have designed the experiments after the cultures have sufficiently been established, with several, well-grown axillary buds on the shoot clumps and last subcultured in MS basal medium. We put 2 clumps each having 6-8 shoot buds per culture bottle and recorded shoot multiplication after 14 /21 days. It was observed that media combination C15 (MS + BAP 1.0 mg/lt + Kn 1.5 mg/lt) and C6 (MS + BAP 0.5 mg/lt + Kn 0.5 mg/lt) have recorded maximum shoot proliferation rate as compared to the other two media. However, C15 combinations gave the highest number of shoots per jar (after 14 days of inoculation). In both the cases, the shoots formed were showing moderate growth in height but number of shoots per jar were significantly higher than the other media combinations . The number of shoots had achieved a multiplication rate of 3.26 in C15 and 3.1 in C6 (after 14 days). It is further observed that there was no significant difference between the number of shoots generated between 14 days and 21 days of each treatment. Pawar, S. V. *et al.*, 1997 also observed that effect of growth regulators on in vitro multiplication of sugarcane cultivars (Co-86032, Co-740 and Co-8014). They had designed the experiment to study the effect of different levels of kinetin, benzyl-aminopurine (BAP) on shoot multiplication, length of main shoot and number of leaves on main shoot on MS medium was supplemented with kinetin and BAP. The highest shoot multiplication ratio for all cultivars was recorded in the MS medium supplemented with 1.5 mg kinetin/lt, 1.5 or 2.0 mg BAP/lt.

In control experiment, using only MS basal medium, rate of shoot multiplication is significantly lower than any other media combinations using cytokinin. It essentially indicates that use of cytokinins have a positive role in multiplication of Sugarcane. Since all the cultures were incubated under the same set of conditions of light and humidity but they responded differently to different media combinations so it proves that both BAP and Kinetin have an important role to play in the axillary shoot proliferation in the multiplication stage of the experiment. Here the results also matched with results obtained by Pakistan by Kazim Ali; Shahid Afghan (2001) who reported that Basal medium (MS) supplemented with benzyl amine purine [benzyladenine] (BAP) and kinetin (K) was used for rapid shoot multiplication. Though we had gone for sixteen different combinations of the medium but the above mentioned four combinations had shown the good response for the multiplication stage and hence are discussed here.

Table 5

Observation of different media combinations for shoot length:

Each culture jar : 2 clump each having 6-8 shoots on inoculation day

No of replicates: 3

Media combination	Av shoot height / jar on inoculation	Av shoot height / jar on 14 days	Av shoot height / jar on 21 days
Control , C0 MS Basal	3.08 ± 0.2	3.88 ± 0.4	4.01 ± 0.2
C6: MS + 0.5 BAP+0.5 Kn	4.03 ± 0.2	5.9 ± 0.1	6.13 ± 0.1
C8: MS + 1.5 BAP +0.5 Kn	5.0 ± 0.3	8.2 ± 0.5	8.6 ± 0.1
C13: MS + 0.2 BAP+1.5 Kn	4.7 ± 0.2	7.8 ± 0.4	8.03 ± 0.4
C15: MS + 1.0 BAP+1.5 Kn	4.13 ± 0.3	5.9 ± 0.2	5.9 ± 0.1

For observation of shoot height for different media combinations, we found that C8 (MS + BAP 1.5 mg/lt+ 0.5 mg/lt Kn) has shown maximum shoot height increase. Other media combination C13 (MS + 0.2mg/lt + 1.5 mg/lt Kn) has also shown promising results.

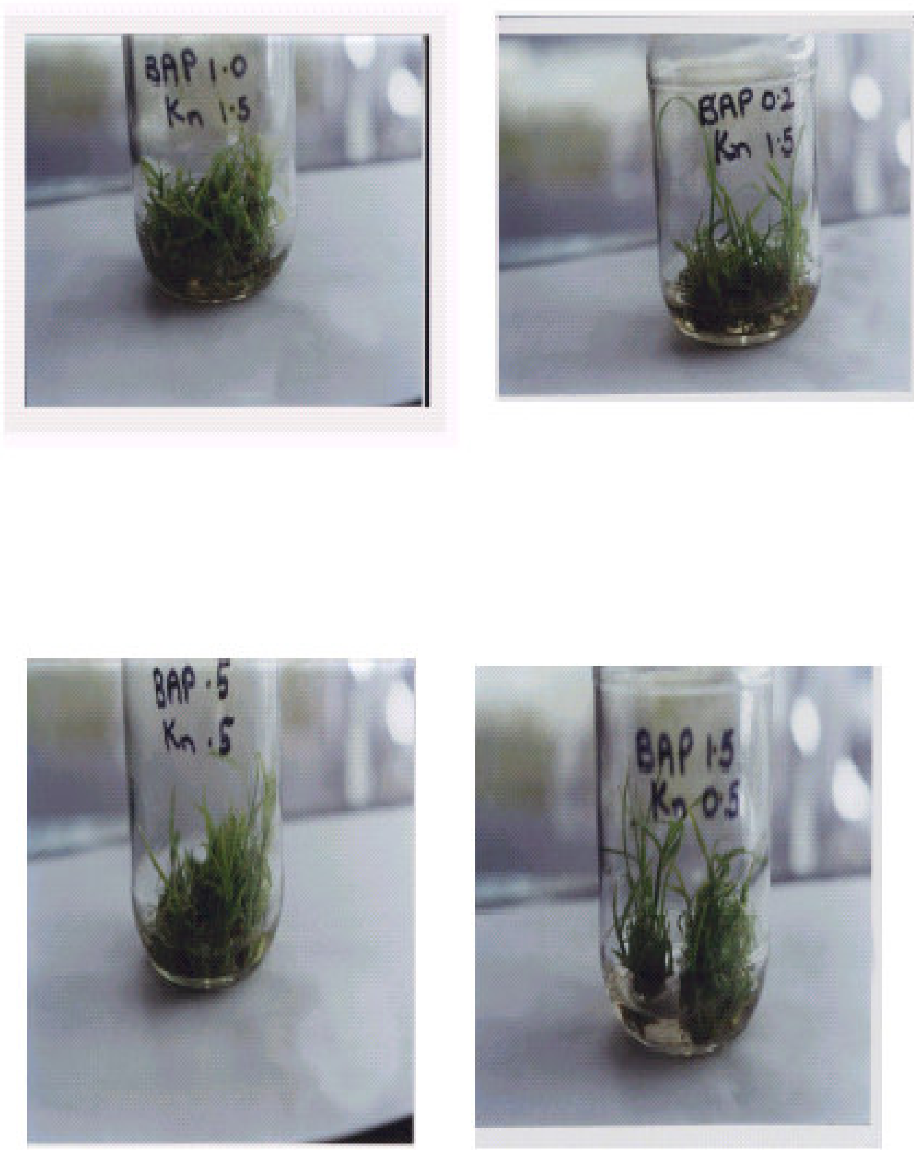


FIGURE SHOWING MULTIPLICATION STAGE OF SUGARCANE IN DIFFERENT COMPOSITION OF MEDIUM

6.c. ROOTING RESULTS

After 2-3 multiplication subculture, when the cluster size and shoot height attains optimum size, we inoculated the shoot clumps into one cycle of MS basal medium to reduce cytokinin carry over effect and then put into different rooting media combination. We have carried out the experiments in several combinations, but the best results obtained have been shown here for reference. Here as well, we inoculated 2 clumps each having 6-8 shoots and take rooting observation on 14 days.

Table 6

Observation of rooting of shoots

Each culture jar: 2 clump each having 6-8 shoots on inoculation day

No of replicates: 3

MEDIUM	Av. height of 5 shoots (14 days)	No. of rooted shoots (14 days)	Av. root length (14 days)
A) 5 NAA (Full strength MS) + 70gm/lt Sugar	8.6 ± 0.2	13.33 ± 0.6	3.86 ± 0.1
B) 3 NAA+ 2 IBA (Full strength MS) + 70 gm/lt Sugar	7.66 ± 0.2	11.67 ± 0.6	3.73 ± 0.06
C) 0.5 IBA (Half strength MS) + 30 gm/lt Sugar	11 ± 0.1	10.67 ± 0.6	2.86 ± 0.1

In case of rooting experiments, the best results were obtained in the medium that was supplemented with MS + NAA (5mg/l) and which had elevated levels of sugar (70gm/l). It was found that roots initiate after 8-10 days and sufficient rooting was obtained within 14-15 days after inoculation into the medium. These newly formed roots showed good growth and were slightly white to pinkish in colour and normal in appearance. The roots were approximately 3.8 cm in length and rooting percentage is approximately 85% for the same medium. It was important to note that most of the shoots that were inoculated into the medium showed considerable root formation within 15 days. Some of references cited here have also indicated that the use of NAA is desirable for the best response to rooting in case of sugarcane. (Singh, B. Yadav, G. C. Lal, M., Sugar Tech, 2001). Another observation study was found that medium containing same concentration of plant growth regulator (5 mg/l NAA) but with 3% sugar showed relatively less rooting as compared to medium containing 7% sucrose. It essentially proves that higher sugar concentration has a positive impact on the root formation. This result is in accordance with the result obtained by Mitchell, S. in which he performed in vitro performance of a range of sugarcane clones evaluated in the Jamaican variety development programme at Sugar Industry Research Institute (SIRI), Mandeville, Jamaica. However, 72% rooting is observed in the medium which had 3 mg/l NAA + 2mg/l IBA with 70 gm/lt of Sugar. It was also observed that approximately 66% rooting was observed in the medium containing solely indole butyric acid (0.5mg/l) with half strength MS medium, but one important observation is the average shoot height has increased significantly. This result for rooting using IBA was also in accordance with the work done in Pakistan by Kazim Ali and Shahid Afghan in which micropropagation of 8 sugarcane clones was studied using meristem tip culture method in which half strength of MS + indole butyric Acid (IBA) was used for induction of roots. Though it is reported that several of the other cultivated species show sufficient rooting in MS basal medium only but in our case, control experiment (RT_0) having no phyhormones failed to generate roots. In the present study we found that IBA plays a pivotal role in the induction of rooting in the plants grown in the tissue culture lab.

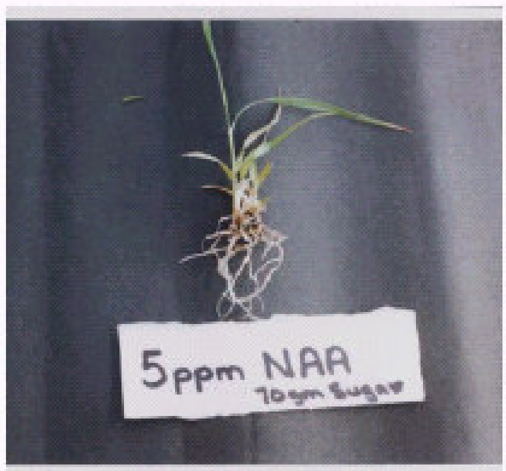


FIGURE SHOWING ROOTING STAGE OF SUGARCANE PLANTS

6.d. TRANSPLANTATION TO THE POLYBAGS

After the sufficient rooting of the sugarcane cultures they were then transferred to the polybags that were having peat and then watered adequately. Due to the lack of time we were not able to find out the proper hardening of the sugarcane plantlets.

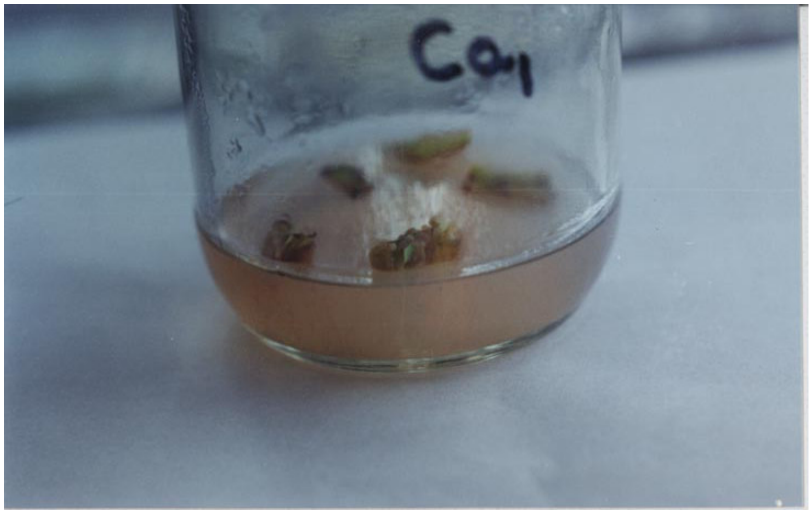
TRANSPLANTATION OF THE PLANTS TO THE POLYBAGS

6.e. CALLUS INDUCTION RESULTS

Callus initiation generally requires auxin and cytokinin or both in the nutrient medium. The leaf and the spindle segments inoculated on the MS basal media with different combinations and concentrations of 2,4-D. In some of the combinations 10% coconut water was also added in the medium. The cultured bottles showed the initiation of callus after the period of 3 weeks. Initially the explants were found to be increased in size. Callus initiation began from the cut ends of the explant that is in contact with the medium. After the induction of the callus from the base of the explants they showed good growth and entire surface of the explants were totally covered with light rose to brown white callus tissue within the next 1-2 weeks. In the present study 4mg/l 2,4-D and 2,4 –D + coconut water (10%) showed the development of the callus but the size of the callus formed in the medium containing coconut water was comparatively less in size as compared to medium containing 2,4- D only. Ajinder Kaur *et al*, 2001 has reported different factors affecting efficient plant regeneration from long-term maintained callus cultures were investigated in three sugarcane cultivars, viz. CoJ 64, CoJ 83, and CoJ 86. The medium Murashige and Skoog (MS)+2,4-D (4 mg/litre)+BAP [benzyladenine] (0.5 mg/litre) induced excellent callusing in the spindle explants of all the three cultivars .On the other hand the explants cultured on the basal medium without any phytohormone failed to produce any response at all. It was also observed that medium with low level of concentration of 2,4-D (2mg/l) produced comparatively less response. From the above result it was found confirmed that 2,4-D is the crucial factor in the induction of the callus from sugarcane explants.

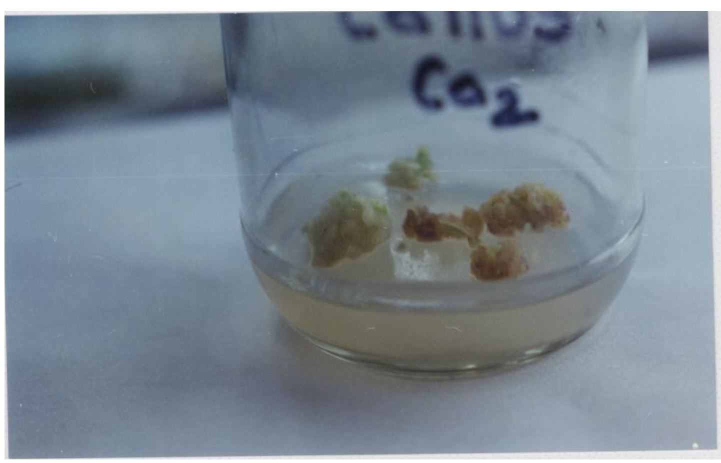
DNA ANALYSIS

For the analysis of the genetic comparison of the normal and regenerated plants aimed to identify any possible somaclonal variants of the plant we did the DNA analysis also. But we were able to complete only the first of this work that is the isolation of DNA from normal plants.



CALLUS INDUCTION IN MEDIUM CONTAINING MS+2,4-D(4mg/L)+CW(10%)

LEAF CALLUS IN MEDIUM CONTAINING 2,4-D(4mg/L)



CALLUS INDUCTION IN SPINDLE OF SUGARCANE

7.0 Summary and Conclusion

In this present study, we have attempted to work on Tissue culture protocol for Sugarcane, which is an important crop for Punjab farmers. Though there are several problems associated with Sugarcane in relation with cultivation, identification of suitable variety in different agro geographical regions, sale of Sugarcane to the mills, minimum support price for the farmers, and a declining trend of Sugarcane cultivation in Punjab, we hope that with a renewed approach in combination of better package of services, better planting material and combination of Plant Biotechnological approaches for mass propagation will certainly bring a change in the whole scenario. We believe that mass propagation of Sugarcane through Tissue culture approach will definitely result in availability of disease free germplasm, dissemination of newer germplasm with improved agronomic characters for the ultimate benefit of the farmers.

We observe and conclude the following in our present study:

1. In identifying the sterilization protocol for sugarcane explant(var CoJ 85), our experience with the followed protocol is satisfactory .
2. We have also observed that the explants have to be subcultured for 3-4 times (in 7-10 days interval) for at least 4 weeks in solid medium before transfer to the liquid medium for further establishment. The media combination used for initial establishment phase is MS Basal + 0.5 mg/lt BAP + 0.5 mg/lt Kn + 30 gm/lt Sugar + 8.0 gm/lt agar.
3. At the multiplcation stage, we found that two combinations MS + 1.0 mg/lt BAP + 1.5 mg/lt Kn + 30 gm/lt Sugar and MS +0.5 mg/lt BAP + 0.5 mg/lt Kn + 30 gm/lt Sugar has shown best results for multiplication rate. However, at this stage, shoot elongation has been found to be maximum in MS + 1.5 mg/lt BAP and 0.5 mg/lt Kn + 30 gm/lt.
4. Significant rooting response found in media combinations having MS + 5 mg/lt NAA + 70 gm/lt sugar and as compared to the media with same conc of Auxin but reduced sugar(30gm/lt) has a reduced response for rooting.
5. We have transplanted a few plants for hardening but due to time constraints we could complete the experiments. To standardize a

successful Tissue culture protocol, successful hardening of microshoots are very essential and further experiments are needed.

6. For callus induction, MS + 2,4-D 4 mg/lt is found to be satisfactory, however, we could not experimented further with the callus growth.
7. In order to ascertain the true to type character of the Tissue culture raised plantlets through axillary shoot proliferation, it is essential to check at the genomic level, it is felt that further experimentation is needed towards achieving the result.

BIBLIOGRAPHY

- **Agnihotri(1998).** Evaluation of fungal and bacterial antagonists against *Colletotrichum falcatum*, causing red rot of sugarcane in Sugarcane and its problems. Indian sugar oct.2000 423-425
- **Ajinder Kaur, 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**, No.2 : 167-172, 6 ref.
- **Barber (1931).** History and development of sugarcane as a world crop . in *The Sugarcane*:1-5.
- **Bhau, Brijmohan Singh (1999).** Hairy root culture and secondary metabolite production. Role of biotechnology in medicinal and aromatic plants, **Vol II**, Ukaaz Publications, Hyderabad.
- **Bhojwani and Razdan (1992).** Plant tissue culture theory and practice Elsever publication
- **Brandes (1985).** History and development of sugarcane as a world crop . in The Sugarcane:1-5.
- **Cheema, G. S. *et al* (1995).** Current Science,68, **6** : 650-653.
- **Chengalrayan, K, Gallo-Meagher, M. (2001).** Effect of various growth regulators on shoot regeneration of sugarcane. *In Vitro Cellular & Developmental Biology*, **37** : 434-439.
- **Dhumale, D. B, Ingole, G. L Durge, D. V. (1994).** In vitro regeneration of sugarcane by tissue culture. *Annals of Plant Physiology*, **8** : 192-194.
- **E.C. Cocking.** Somatic embryogenesis and plant regeneration from rice protoplasts Plant Genetic Manipulation Group, Embryogenesis in Barny yard grass, *Plant science* : 161-163.
- **Enriquez, G. A., Arencibia, A. Selman-Housein, G. Hernandez, L. Tiel, K., Fuentes, A. D. (2001).** *Sugarcane biotechnology and genetic engineering experience at CIGB*: 578-579.

- **Evans, D.A, Sharp, W.R, Ammirato, P.V. (1986).** *Handbook of Plant Cell culture* , 419-456. Macmillan Publ. Co., New York.
- **Gahukar, S. J, Jambhale, N. D. (2000).** Callus induction and regeneration in Saccharum cultivars as influenced by mutagen treatments. *Journal of Maharashtra Agricultural Universities* **25** : 219-220.
- **Gamborg O.L, Phillips G.C. (1998).** *Plant cell, tissue and organ culture* , Narosa Publ. House, New Delhi.
- **Geetha S, Padmanabhan D. (2001).** Effect of hormones on direct somatic embryogenesis in sugarcane, *Sugar Tech* **3**: 120-121.
- **Gottlieb Harberlandt, (1902).** " Experiments on the culture of isolated cells".
- **Govil and Gupta (2000).** *Biotechnology in horticulture and plantation crops,* : Eds. K.L.Chadha et. al., Malhotra Publ. House, New Delhi.
- **Guha, S., S. C. Maheshwari (1958).** Cell division and differentiation of embryos in the pollen grains of Datura in vitro, *Nature* 212 : 97-98.
- **Gupta, J. N (1995).** Current Science.**68**, No.6: 650-653
- **Harrison, D, Waldron, J, Ramage, C, Peace, C., Cox, M. , Birch, R. G. Carroll, B. J.E.D. Hogarth, D. M. (2001).** New DNA technologies provide insights into the molecular basis of somaclonal variation in sugarcane: 580-581
- **Hartmann and Kester**, 1975; Morel, 1975; Rao, 1977 http://www.alexsoftware.com/cns/LastNames.txt
- **Horsch, R.B., Fraley, R.T., Rogers, S.G., Sanders, P.R., Lloyd, A. and Hoffmann, N. (1984).** Inheritance of functional foreign genes in plants. *Science*, **223** : 496-498.
- **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** : 8.
- **John Hewitson.** Science and plants for schools (website)

- **Kazim Ali and Shahid Afghan (2001).** Pakistan Sugar Journal, 2001, 16(6): 11-14, 14 ref.
- **Khan Irfan A, Khanum Atiya (1999).** Role of biotechnology in medicinal and aromatic plants, vol. II, Ukaaz Publication, Hyderabad.
- **Khanna, P. (1982).** Tissue culture and useful drugs. A review of 38 plant species grown in vitro. In cultivation and utilization of Medicinal and aromatic plants, Eds. C.K. Atal and B.M.Kapur., RRL Jammu Tawi, pp. 824.
- **Kharinarain, R. P (1996).** Russian Journal of Plant Physiology: **43**, No.1, 97-100.
- **Krishnamurthy and Tlaskal (1999).** *Bharatiya sugar*.
- **Lal, N. Lal, N.; Singh, H. N. AA : G.S. (1993) :** Sugar Cane, 1993(**2**) :2-3, 14 ref.
- **Liu et al (1983).** *Bharatiya sugar*, 1999
- **Maribona *et al* (1986).** Bharatiya sugar, 1999
- **Mascarenhas A. F. (1997).** Hand book of plant tissue culture.
- **McCoy, T and Phillips, RL. (1982).** *Can J Genet Cytol* **24**:559-566.
- **Mitchell, S. (1997).** *Proceedings of the West Indies Sugar Technologists 26th Conference*, 22-26 September 1997: 80-87.
- **Mohatkar L.C. (1993).** Current Science:**64**, No.8, 604-605.
- **Muir, W. H., A. C. Hildebrandt, A. J. Riker (1954)**. Plant tissue cultures produced from single isolated cells. *Science*, **119** : 877-878 .
- **Mulleegadoo, K. D. ; Dookun, A. (1999)** Revue Agricole et Sucrière de l' Île Maurice: 78, No.2, 35-39.
- **Murashige, T. & Skoog, F. (1962)**. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. **15**:473-497
- **Nadgauda, R.S.**, *et al* , NCL WEBSITE
- **Nagata, T. and I. Takebe (1970).** Cell wall regeneration and cell division in isolated tobacoo mesophyll protoplasts. *Planta* **92**:301-308

- **Naik, G. R. (2001)** Sugarcane biotechnology. BT- Sugarcane biotechnology , pp. 165-168)
- **Naritoom, K. Sooksathan, K. Korpradittskul, V. Suksathan, I. isessuwan, R. Burikam, S. Klingkong, S. Suriyachaiyakorn, M. (1993).** Plant regeneration from shoot tip culture of sugarcane. *Kasetsart Journal of Natural Sciences* **27** : 286-291
- **O.L. Gamborg and G.C. Phillips eds. (1995)**. Embryo culture / embryo rescue for wide cross hybrids. *Textbooks for Training, Education and Research in Applied and Basic Plant Biotechnology*. Vol 1.
- **Padmanabhan et. al. (1996) in "**Evaluation of fungal and bacterial antagonists against *Colletotrichum falcatum*, causing red rot of sugarcane in Sugarcane and its problems" by Senthil, N, Jayaraj .J, Shanmugham". *Indian sugar* **Oct 2000** : 423-425
- **Patel, A. A., Patel, S. R. Patel, C. L., Prajapati, B. S. (2001).** Effect of media composition on in vitro multiplication of sugarcane varies. *Indian Journal of Genetics and Plant Breeding* **61** : 82-83.
- **Pawar, S. V, Patil, S. C., Jambhale, V. M. Naik, R. M. Mehetre, S. S. (1996).** Rapid multiplication of commercial sugarcane varieties through tissue culture. *Indian Sugar* **52**, 183-186.
- **Pawar, S. V., Patil, S. C., Ambhale, V. M, Naik, R. M., Mehre, S. S. (1996).** Rapid multiplication of commercial sugarcane varies through tissue culture. *Indian Sugar* **52** :183-186.
- **Sanford, J. C. (1990).** Biolistic plant transformation. *Physiologica Plantarum* **79**: 206-209.
- **Schieder, O. and Vasil, I. K. (1980).** Protoplast fusion and somatic hybridization. *Int. Rev. Cytology*, **Suppl 11B**: 21-46.
- **Shahi. H.N. (2002).** *The Hindu Survey Of Indian Agriculture*.
- **Shahid, M. T. H. Sajid-ur-Rehman, Shaheen, M. S, Javed**. **(2001).** Variability studies in sugarcane (*Saccharum officinarum L*) somaclones. *Pakistan Sugar Journal* **16** : 27-31.

- **Singh, B, Yadav G. C, Lal M. (2001).** An efficient protocol for micropropagation of sugarcane using shoot tip explants. *Sugar Tech.* **3** : 113-116
- **Singh, B.D.** *Plant Breeding-principles and methods*. Vol 2003 Kalyani publishers, Ludhiana.
- **Skirvin, R.M., Janick (1976).** Natural and induced variation in tissue culture. *Euphytica* **27**:241-266.
- **Skoog and Miller,** Plant Tc listserv.
- **Sorory, A, Hosien-Zadeh, A. (2000).** Iranian Effects of genotype, media and explant on callus initiation and plant regeneration in sugarcane. *Journal of Agricultural Sciences* **31**: 211-220
- **Sreenivasan, T. V, Sreenivasan, J. (1992).** Micropropagation of sugarcane varieties for increasing cane yield. *SISSTA Sugar Journal* **18**: 61-64.
- **Takebe, I, G. Labib and G. Melchers**. **(1971).** Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. *Naturwiss* 58, 318-320.
- **Vasil, I. K. and V. Vasil**. **(1980)** Isolation and culture of protoplasts. *Int. Rev. Cytology* **118** :1-19.
- **Viswanathan *et al* (1997)** Evaluation of fungal and bacterial antagonists against *Colletotrichum falcatum*, causing red rot of sugarcane in Sugarcane and its problems. Indian sugar **Oct. 2000** : 423-425
- **Viswanathan, Padmanaban (1998).** *Indian sugar*.
- **Welander,T.(1977).** In vitro organogenesis in explants from different cultivars of Bigonia. *Physiology Plant* **64** : 142-145.
- **Xiao Guan Li, Yang Qing Hui, Li Fu Sheng, Yang Sheng Chao (2001).** Study on the relationship between endogenous hormones and the rooting rate of plants in subculture of sugarcane. *Journal of Yunnan Agricultural University* **16**: 271-273.