MICROPROPAGATION IN SUGARCANE
(*Saccharum* spp. hybrid)

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By

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CERTIFICATE

This is to certify that the thesis entitled **"MICROPROPAGATION IN SUGARCANE (*Saccharum* spp. hybrid)"** submitted by Mr. **GANAPATI HEGDE** for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **GENETICS AND PLANT BREEDING** of the University of Agricultural Sciences, Dharwad, is a record of research work carried out by him during the period of his study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar titles.

DHARWAD
AUGUST, 1998

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AFFECTIONATELY DEDICATED

TO MY

BELOVED PARENTS

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INTRODUCTION

I. INTRODUCTION

Sugarcane, grown primarily for sugar, is cultivated throughout the tropics and subtropics (35° N to 35° S) on a variety of soils. It has been adapted to a wide range of environments and cultural practices. A very versatile tropical grass, it belongs to tribe Andropogonae of the family Poaceae and supposed to have originated in New Guinea (Liu, 1984).

Sugarcane is an important commercial crop. On a world-wide basis 60 per cent of the sugar and in India almost all of it, is obtained from sugarcane. Presently, it is cultivated on an area of about 18.318 million hectares producing 1147.9 million tonnes of cane (Anon., 1995). The principal sugarcane growing countries are: India, Brazil, Cuba, Australia, Mexico, United States of America, Phillippines and China.

Although sugar is the most important product of sugarcane, various by-products serve as industrial raw material. Bagasse is used as fuel in the sugar industry itself as a fuel and paper, pulp and board are also made out of it. Molasses is an energy rich by-product of sugar manufacture. It is used in the alcohol, citric acid and animal feed industries. The green tops of the plants obtained during harvest and thinning operations are valuable green fodder for cattle in the rural India.

In India, sugarcane is grown all over the country except in the extreme north and high altitudes. It is an important cash crop, second only to cotton. Though sugarcane occupies about two per cent of the gross cropped area, it contributes to about seven per cent of the gross value of agricultural output. In 1995, it was grown on 3.75 million hectares with a production of 259 million tonnes. About 50 per cent of the cane was processed into white sugar and the remaining was used for producing jaggery, khandasari sugar and for seed purposes (Anon., 1995).

There are two sugarcane belts in the country with distinct agroclimatic conditions. The tropical belt comprises of Maharastra, Tamil Nadu, Karnataka, Andhra Pradesh, Gujarat and Kerala. The sub-tropical belt, where a large part of the cane area lies, comprises of Uttar Pradesh, Punjab, Haryana, Bihar and other north and north-eastern states. The yield levels in tropical zone are quite high (90-100 t / ha) compared to sub-tropical zone (40-50 t / ha), against the national average of 65.3 t / ha. The lower productivity in the sub-tropical region is attributed mainly to cooler climate during parts of growing season and the incidence of pests and diseases.

Modern cultivars of sugarcane (*Saccharum* spp. hybrid) are derivatives of interspecific crosses within the genus *Saccharum.* The genetic resources utilised for crop improvement include three cultivated species: *viz*., *Saccharum officinarum* (2n=80), *S. barberi* (2n=82-124) and *S. sinense* (2n=106-120) and two wild species, *viz*., *S. spontaneum* (2n=40-120) and *S. robustum* (2n=60-80). The process of introgressive hybridisation, known as 'nobilisation', between *S. officinarum* and other *Saccharum* spp. has resulted in varieties with improved yield, sucrose content, disease resistance and wide adaptability (Liu, 1984).

Most of the present commercial cultivars are highly heterozygous, cytogenetically complex interspecific aneuploids with chromosome numbers ranging from 2n=100 to 2n=130(Sreenivasan *et al*., 1987b) with high degree of autoploidy. The vegetative propagation normally ensures stability of the chosen cytogenetic features and plant characters in sugarcane. However, care is needed to maintain the pure stock, free from diseases and pests.

Sugarcane is propagated from setts made from 7-8 month old canes and each sett consists of 2-3 nodes. This type of planting material can carry pathogenic microbes, virus and mycoplasma. Therefore, it is necessary to source the seed cane from healthy gardens. Since a large number of sets (40 - 50,000/ha) are required, it is often difficult to ensure genetic purity and freedom from pathogens, even after seed treatment with chemicals and steam therapy. It has been suggested long back that micropropagation, starting with meristem culture, be used as a part of the seed multiplication programme. But the economic considerations have come in the way of adoption of this technology (Hussey, 1986). While it appears to be so under normal circumstances, there is increasing awareness about the benefits of micropropagated stock among the cane growers in areas with preponderance of red rot, mosaic, grassy shoot and smut diseases.

Although many studies have been made on micropropagation in sugarcane, only in a few reported cases full protocols are available. Keeping all these points in view, the present investigation on sugarcane micropropagation was undertaken on three popular cultivars: 'Co 8014', 'Co 85002' and 'CoC 671' with the following objectives:

1. Standardisation of protocols for the micropropagation of popular sugarcane cultivars of the region.
2. Development of alternative media inputs to economise micropropagation.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Plant tissue culture is an important tool in both basic and applied studies as well as in commercial applications. The technology owes its originto the ideas of Haberlandt who suggested that it should be possible to cultivate vegetative cells artificially. He introduced the concept, cell totipotency, that all living cells containing a normal complement of chromosomes should be capable of regenerating plants. Although Haberlandt was not successful in providing experimental proof, tissue culture studies during 1950s and the 1960s proved the totipotency of the cells and firmly established techniques for the cultivation of organs, tissues, cells and *in vitro* morphogenesis (Thorpe,1990).

Early tissue culture studies were centred around a few dicotyledonous species such as *Datura, Nicotiana* and carrot, which were highly responsive. Even now, they are considered as model systems for tissue culture studies. However, the monocotyledonous species proved to be recalcitrant. A major breakthrough in tissue culture of monocots, especially cereals and grasses, occurred when high salt Murashige and Skoog medium (1962) was formulated. Developed for tobacco pith callus for better growth than in existing low salt media, it also supported growth of most of monocots. Soon the tissue culture techniques were applied to many of monocots and dicots species.

Sugarcane tissue and cell cultures were initiated for physiological studies in 1961 in Hawaii (Nickell, 1964). With the demonstration of totipotency in grasses (Urata and Long,1968) and the occurrence of chromosomal mosaics in sugarcane (Heinz *et al*,1969), serious efforts were made to show totipotency in sugarcane callus cultures and then to produce and isolote somaclone for improved agronomic characteristics (Heinz and Mee, 1969; Barba and Nickell,1969). The success at Hawaii, led to cell and tissue culture work in Fiji (Krishnamurthi, 1974) and Taiwan (Liu,1971) to isolate disease resistant and other useful variants. Research Institutes in Australia, India, Florida, Philippines, Brazil and France have also investigated sugarcane tissue and cell cultures. In fact, most research stations engaged in sugarcane improvement include one or more aspects of tissue culture as a supplementary component to conventional breeding practices (Maretzki, 1987).

The literature available on various aspects of *in vitro* culture studies of *Saccharum* spp. with special reference to micropropagation is reviewed here.

2.1 CALLUS INDUCTION AND MAINTENANCE

Modified Murashige and Skoog formulation (1962) is generally used as the culture medium for callus induction, maintenance and routine manipulation. Withdrawal of 2,4-D from the medium makes it suitable for plant regeneration (Larkin, 1982).

Most investigators have preferred young leaves and subapical meristems as explants. (Heinz *et al*., 1977; Larkin, 1982; Sreenivasan and Jalaja, 1983; Irvine, 1984; Liu, 1984; Chen *et al*., 1988). In addition, young inflorescence (Heinz and Mee,1969), roots (Sauvaire and Galzy, 1980) and mature caryopsis (Burner, 1992) have also been employed for callus initiation. In all these studies 2, 4 - D at 1 - 3 mgl^{-1} has been used for callus induction and maintenance.

Callus growth in sugarcane usually proceeded rapidly and required subculture every 3-4 weeks. Generally, callus is further subcultured on the same modified MS medium with 3 mgl^{-1} 2, 4 - D (Heinz *et al.*, 1977; Liu, 1981). The morphogenic capacity of the tissues after few months in a culture, declines rapidly (Conger,1981). The loss of morpogenic potential has restricted the effective utilisation of tissue culture techniques for genetic improvement. However, plants can still be obtained from callus cultures maintained for as long as two years (Dobariya, 1994).

Among various callus types produced by the members of poaceae, the embryogenic callus, widely used for long term culture and regeneration, is described as slow growing, compact, nodular in shape and white to yellowish in colour. At cellular level it is composed of small, isodiametric, densely cytoplasmic and rapidly dividing cells (Lu and Vasil, 1982; Ozias - Akins and Vasil, 1982; Green, 1982). Taylor *et al.* (1992) obtained four types of callus tissues in sugarcane but, only embryogenic and organogenic calluses where capable of producing plants. For establishment and maintenance of embryogenic cultures in sugarcane, lower levels (0.5 - 1.5 mgl^{-1}) of 2,4 -D (Ho, 1982; Ho and Vasil, 1983) and dark incubation (Ho and vasil,1983 ; Chen *et al.,* 1988) were essential for long term cultures.

Long term maintenance and subsequent regeneration of plants from sugarcane callus cultures was achieved by selecting green regenerative sector of callus (Fitch and Moore, 1990). They employed modified MS medium with 3 mgl^{-1} 2,4 - D for callus initiation and maintenance on the same medium with 0.5 mgl^{-1} piclorom instead of 2,4 - D . They demonstrated the retention of morphogenic pontential even after four years of culture, following selective subculture of green sectors. Chen *et al.*

(1988) found that white compact callus was capable of regenaration. Using such a scheme, it is possible to maintain the regeneration capacity in several sugarcane cultivars for many months, after callus induction.

2.2 MORPHOGENETIC STUDIES IN SUGARCANE

Sugarcane callus and cell cultures could be effectively switched on to a morphogenetic pathway, for plant regeneration through organogenesis or embryogenesis,by complete removal or lowering the auxin levels (Heinz and Mee, 1969; Liu 1981). However, the frequency of regeneration depended upon cullture type and age, medium composition and the genotype used (Liu; 1984; Chen *et al.*, 1988). Nadar *et al.* (1978) reported somatic embryogenesis from cultured leaf sheath on modified MS medium with 3 mgl^{-1} 2,4 - D. Histologcal examination of the callus showed different stages of embryoids in the presence of the 2,4 -D. Plant differentiation occurred only in the absence of auxin. Embryogenic cell initiation required high auxin levels.

A convincing evidence for embryoid formation in sugarcane was reported by Ho and Vasil (1983). They demonstrated the formation of somatic embryoids within 33 - 35 days when callus was grown on MS medium containing 0.5-3.0 mg^{-1} 2,4 -D. The histological examination of the cultures revealed a clear bipolar organisation of embryoids with a distinct scutellum, coleoptile and coleorrhiza. In many of the embroyoids, the root meristem was either absent or its development was delayed. Chen *et al.* (1988) reported plant regeneration *via* both organogenesis and somatic embryogenesis in sugarcane callus cultures. Compact morpogenic callus, younger than twelve months, formed somatic embryos and / or shoot meristems when transfered to a medium with activated charcoal. With an increase in the age of the callus, somatic embryogenesis decreased and organogenesis was predominant in older culture.

Recently, Brisibe *et al.* (1993) followed the formation of embryogenic callus and growth of embryos of sugarcane through the electron microscope. The somatic embryos passed through distinct stages similar to those of grass zygotic embryos including bipolar embryonic orientation with clear embryo axis and scutellum. Higher accumulation of major storage reserves was also evident in both the tissues.

2.3 SOMACLONAL VARIATION IN SUGARCANE

2.3.1 Nature of somaclones

Heinz and Mee (1969) at the Howaiian Sugar Planters Association were the first to report morphological and cytological variations in callus derived plants of *Saccharum* spp. Morphological variation was observed for plant type, leaf attitude, tillering and errectness. One of the clones with distinct morphological changes, had chromosome number 2n = 117 to 124 compared to 2n = 106 to 107 in the donor. It was also demonstrated that the suspension cultures of several cultivars maintained for more than six years had variation in chromosome number for all the five clones examined (Heinz *et al.*, 1969). It is thought that opportunities for yield increase can be created by producing chromosome number variants in existing cultivars (Sreenivasan *et al.*, 1987a).

The chromosome number of the sugarcane cells has been doubled by treating cell suspensions with colchicine (Heinz and Mee,1970) and the somaclones with chromosome number ranging from 150 to 225 were produced from numerous species and hybrids. Some of these plants developed from intra and inter specific hybrids were vigorous (Heinz *et al.,* 1977).

Liu *et al.* (1977) reported that in colchicine free suspensions of 'F-164' (2n=108), chromosome number varied from 2n = 92 to 191. But, when treated with colchicine, number increased to as many as 309 per cell. A number of plants regenerated from colchicine treated suspension cultures of 'H 69-9092' (2n =122) were aneuploid (Nagai *et al.*, 1986). This provided an opportunity to critically analyse the effect of changes in chromosome number on plant morphology. There were significant negative correlations between chromosome number and most of the morphological traits and several somaclones with low chromosome number were vigorous. It appears that present day sugarcane cultivars already have too many chromosomes.

2.3.2 Isolation of desirable somaclones

During 1970s, the work on sugarcane tissue culture was started in Fiji particularly to isolate plants resistant to Fiji disease caused by a leaf hopper transmitted virus (Krishnamurthi, 1974). While screening the somaclones for their reaction to Fiji disease and downy mildew (*Sclerospora sacchari*), they found some somaclones with increased resistance for both the diseases. Further evaluation of these somaclones demonstrated the stability of resistant phonotypes (Heintz *et al.*,1977; Krishnamurthi, 1981,1982). Somaclones with varying level of resistance to eyespot disease caused by *Helminthosporium Sacchari* were also isolated (Heinz, 1973; Heinz *et al.*, 1977).

Studies conducted at the Taiwan Sugar Research Institute (Liu, 1971; Liu *et al.*, 1972) have shown wide spread occurrence of variation for many morphological characters including stooling and erectness among sugarcane somachlones. In a subsequent study, Liu and Chen (1976) examined 417 somaclones from eight varieties for morphology and sugar content. The frequency of mophological changes varied from 1.8 per cent (F 146) to 34.0 per cent (F 156), depending on genotype. The most conspicuous change occurred in auricle length followed by dewlap shape, hair group and leaf carriage. They also observed high sugar content in some of the somaclones, 2 to 12 per cent increase over the donor. In subsequent studies, Liu and Chen (1978) isolated somaclones superior to both the parental clones and the best check (F 160). One of them was better than its donor (F 164) for cane yeild, sugar yield and stalk number by 32, 34 and 6 per cent, respectively. The same somaclone was also better than 'F 160' by 20,16 and 8 per cent, respectively, for the above characters.

Larkin and Scowcroft (1983) indentified many toxin - tolerant variants among the plants regenerated from callus cultures of variety 'Q 101', highly susceptible to eye spot disease. Sreenivasan and Jalaja (1983) tested 39 somaclones of an Indian variety 'Co 7704' for stay - green character. Normally, the leaves dry between 80 and 120 days but eight out of 39 clones were found to be free from leaf drying. They also reported glabrous leaf sheath variants of 'Co 7717' and somaclones without deep splits in internodes from 'M C 132', while retaining desirable features of respective donors (Jalaja and Sreenivasan, 1988).

Many workers have found that all the variation observed among the somaclones is not transmitted to the next generation, clonal or sexual. Lourens and

Martin (1987) observed more morphological variability among the somaclones regenerated from callus cultures (indirect) than those that did not involve an elaborate callus phase. They also found that most variation was not due to hereditary changes. The reversion of somaclones to original characteristics was less frequent in callus derived somaclones than the others, in a subsequent clonal generation. Sreenivasan *et al.* (1987a) isolated a stable rust resistant somaclone from a susceptible sugarcane variety 'Co A 7601'. The stability of the change was confirmed when 308 plants produced from callus of a resistant somaclone were found to be free from rust infection. Gonzalez *et al.*(1992) also observed wide variations in somaclones of 'C 8751' under rainfed condition. However, the variability for five yield components between the somaclones and donor was not significant.

Fahmy (1990) obtained somaclones resistant to sugarcane mosaic virus (SCMV) from callus cultures of two susceptible varieties. Dobariya (1994) produced large number of somaclones of 'CoC 671' (405) and 'Co 740' (777) from calli. These somaclones showed wide variations for many morphological, yield and juice parameters. For quantitative characters the frequency of variant somoaclones ranged from 9.85 (cane weight / clump) to 48.64 (leaf length) per cent in 'CoC 671' and from 2.96 (number of millable canes / clump) to 32.82 (leaf length) per cent in case of 'Co 740', indicating marked genotypic differences.

2.3.3 Somaclonal variants in intergeneric hybrids involving sugarcane

The phenomenon of chromosomal rearrangement during tissue culture has the potential to be exploited for introgressing specific genes from wild relatives into modern cultivars. In sugarcane, interspecific and intergeneric hybrids have been produced (Price,1967) and successfully used in sugarcane improvement and phylogenetic studies (Sreenivasan *et al.,* 1987a). Sreenivasan and his group at Sugarcane Breeding Institute, Coimbatore, India have pioneered the work on somaclonal variation in intergeneric hybrids. Sreenivasan and Jalaja (1982) obtained a number of callus derived plants from a hybrid (2n = 52) between *Saccharum* sp. (2n = 80) and *Zea mays* (2n = 20 + 2B). These plants showed marked variations for leaf width, colour and robustness. All plants were also aneuploids with 2n = 48 to 56. Jalaja and Sreenivasan (1987; 1988) obtained a wide array of somaclonal variants from a *Saccharum* sp. X *Sorghum* sp. hybrid for days to flowering, bud sprouting, chromosome number, stalk number, brix, stalk diameter and other characters.

Variation in chromosome number and many morphological characters among somaclones of intergeneric hybrids were also observed by Nagai *et al.* (1991) and Chen *et al.*(1992). Somaclones of *Sacchurum* spp. hybrid (2n = Ca. 115) *X* *Eriathus arundinaceum* (2n = 60), varied widely for most characters studied. Variation in chromosome number from 2n = 66 to 108 and isozyme pattern of many of these somaclones confirmed the genetic basis (Nagai *et al.*, 1991). Similarly colchicine treated cell cultures of three *Saccharum* spp. X *Miscanthus* spp. hybrids produced plants with varied number of chromosomes (Chen *et al.*, 1992).

2.4. *IN VITRO* MICROPROPAGATION

Sexually produced plants begin as a fertilised egg, the zygote, which develops into an embryo containing a shoot meristem. Vegetative propagules of such plants may arise in a number of different ways by reproductive mechanisms that have persisted and diversified throughout the plants kingdom during evolution. These mechanisms which concern the formation and multiplication of shoot meristems, fall into two main categories: (1) the production of axillary meristems by the branching activity of the original shoot apex, and (2) The formation of adventitious meristem, in the form of either shoot apices or embryos, from the cells and tissues of the plant body. *In vitro* techniques enable these mechanisms to be exploited with very high efficiency, either by releasing the natural controls on axillary meristems formation or by providing suitable conditions for the regeneration of adventitious meristems from orignal or culltured somatic cells.

The distinction between the two main pathways of vegetative reproduction is that the genetic stability of the propagules during multiplication is more and their susceptibility to mutation or any form of genetic changes is less in case of axillary shoot proliferation than with adventitious meristems. Studies on mutations have clearly shown that the shoots regenerated adventitiously from parent tissue or from callus exibit more mutational changes than axillary shoot apices, thus confirming the relative stability of the existing, organised meristems (Broertjes and Van Harten, 1978). The multicellular layer of the shoot apex will protect it to a considerable degree from being taken over completely by mutant cells by forming predominantly unstable chimeras and occasional periclinal chimeras. Diplontic selection and more efficient DNA repair mechanism of the cells in the extreme tip of the apex also contribute towards genetic stability.

It is evident from the foregoing information that in order to ensure genitic uniformity of plants propagated by tissue culture, axillary shoot proliferation should be used wherever feasible. Precautions are obviously necessary to avoid in advertant recycling of mutant shoots and using levels of growth regulators that might induce adventitious shoots and callus. *Asparagus* multiplied by axillary shoots by Murashige *et al* .(1972) were true to type, while those regenerated from callus by Malnassy and Ellison (1970) include up to 70 per cent polyploids.

MICROPROPAGATION IN SUGARCANE

The method of producing large numbers of identical clones by *in vitro* culture is being routinely used for horticultural herbaceous crop plants and ornamentals (Vasil and Vasil, 1980) and increasingly for woody species (Lawson, 1978). Until recently legumes and many grasses resisted propagation *in vitro* on a large scale, but this situation is changing radically. Commercial sugarcane is a sexually sterile autopolyploid. It is propagated through stem setts consisting of 2-3 nodes, satisfactorily. Micropropagated sugarcane plants are not required routinely for raising commercial crop nor it is cost effective. As such, studies on micropropagation of sugarcane are rare.

At least four areas of applications can be seriously considered for micropropagation of sugarcane : (1) Elimination of systemic pathogens from seed cane before planting, (2) Preservation of breeding stock as juvenile plants, (3) Shipping of disease free varieties and germplasm to eliminate quarantine requirements, (4) Transplanting to replace present vegetative seed plantings.

Three routes to multiplication of sugarcane plants are recognised: (1) Axillary shoot formation, (2) Adventitious shoot formation, and (3) Somatic embryogenesis. Of these axillary shoots are considered by far the safest source for the recovery of identical clones (Vasil and Vasil, 1980). These are obtained from the stimulation of quiescent lateral shoot primordia located in the axil of every leaf or leaf primordia of shoot meristems (Binding, 1975; Murashige, 1978). The relative slowness following this route of multiplication, in many species including sugarcane, is more than compensated for by attainment of genetic uniformity. True axillary bud culture may be extremely difficult to establish in sugarcane but has been convincingly demonstrated by Sauvaire and Galzy (1978) who found it possible to continuously propagate plants form axillary buds of several sugarcane clones. The addition of 6-benzylamine purine (BAP) to the culture medium proved helpful, but required adjustment in concentration depending on the genotype.

Organogenesis *via* adventitious shoot formation required much less stringent conditions for tissue excison. Explanting can be done without the aid of microscope and success rate in achieving organogenesis is high. On the other hand somaclonal variation is the problem. Irrespective of whether the shoots arise through the callus phase or directly form the cells of the cultured organ, the possibility of genetic changes increases.

Coupled with elimination of viruses through meristem culture, micropropagation through axillary bud proliferation is the preferred method in most plant species including sugarcane. The process would serve the twin objectives of eliminating systemic pathogens as well as ensure genetic uniformity of the planting material so generated. Coleman (1970) and Hendre *et al* .(1983b) demonstrated elimination of viruses from infected donor through meristem culture. Sugarcane is listed among species that are amenable to meristem culture to eliminate virus (Mori and Hosokawa, 1977)

Heat therapy in conjunction with bud culture has been used by a number of workers. 'Streak' virus was eliminated in buds of sugarcane following hot water treatment at 59°C for 10 minutes (Roth, 1969) and Ten Houton *et al.* (1968) proposed a combination of shoot tip heat treatment and meritstem culture so that a 10x larger segment of the tissue (1 mm instead of .1 mm) could be used to ensure better survival on nutrient media. Heat therapy in conjunction with bud culture has also been studied by Waterworth and Kahn (1978) as a means for shipping mosaic virus - free plantlets from one country to another country.

Victoria and Guzman (1993) proposed a rapid multiplication method to eliminate systemic pathogens of sugarcane. Fragments of vegetative propagules were treated in hot water (51°C, lh), shooted in a substrate of soil mixture in humid chamber at 41°C and 12h photo period for 20 days. After that, meristems were dissected aseptically and cultured *in vitro* on MS - I medium for establishment. Sreenivasan and Jalaja (1980) at Sugarcane Breeding Institute, Coimbatore cultured directly excised meristems of 0.5 mm length on MS medium to get virus-free plantlents of two cultivars 'Co 62174' and 'Co 7201'. They also obtained virus - free plantlets of 'Co 8338' and 'CoC 671' through meristem culture (Jalaja and Sreenivasan, 1990). Thus meristem culture is known to eliminate systemic pathogens. However, it has been argued that virus elimination may be futile when plants are grown back in areas infested with the pathogens. Yet, healthy and pathogen free planting material wood ensure a clean crop at least to start with.

In addition to elimination of systemic pathogens, the main use of micropropagation is rapid initial multiplication of identified genotypes which otherwise take many years by conventional methods. In most cases this will help in bulking-up a few thousand propagules to serve as an adequate foundation for further conventional increase, thus saving much time, labour and facilities when introducing new plants into cultivation. The actual commercial micropropagation of plants is at present confined mainly to high-value horticultural crops such as ferns, foliage plants and other ornamentals and a few plantation crops. There exist a category of plants where efficient micropropagation protocols are available and yet it is not feasible commercially to supply propogules for raising a crop. Sugarcane is one such plant species where economy in micropropogation would help to popularise tissue cultured plants, especially in areas endemic for certain diseases caused by systemic pathogens.

Hendre *et al.* (1983a) multiplied 'Co 740' cultivar by transferring the established shoot tips to media supplemented with cytokinins. They reported that 200,000 plants could be produced from single shoot tip in 6 months. In another study, Girisham and Buurg (1989) obtained more than 27,500 plants of C.P. 65 - 357 variety after 6 transfers from single shoot tip using MS shoot proliferation medium. Victoria and Guzman (1993) produced 1500 plants per original meristem after 3 transfers.

The cost effectiveness of micropropagation depends on several factors, *viz.*, (1) the rate of multiplication (2) media composition (3) subculturing procedures (4) light and temperature conditions. Especially in the crops like sugarcane, where high density planting is done, the cost per plantlet should be very low so as to popularise micropropogated plants. A major factor is that the labour can account up to 70 per cent of the final cost. Cost reduction methods are required to commercialise the micropropogation (Wang and Charles, 1991).

2.4.1 Culture establishment and multiple shoot formation

The rate of multiplication of the shoot tips is very important to reduce the cost of micropropagated plants. Growth regulators used in media play an important role in achieving desired rate of multiplication.

According to Larkin (1982) MS medium supplemented with 0.5 mg/l IAA and 1 mg/l BA was good for shoot multiplication. In another study the best axillary bud induction was obtained with benzyl adenine (BA) 0.075-0.3 mg/l (Jimenez *et al.*, 1990). Sreenivasan and Jalaja (1980) reported that normal shoot development was obtained from two cultivars 'Co 62174' and 'Co 7201' when cultured on MS medium with 0.05 mg/l BA and multiple shoots were produced when transferred to medium with NAA and kinetin. Alam *et al.* (1995) reported that shoot proliferation was best in sugarcane explants cultured on MS medium containing 0.5 to 1.0 mg BA/l and 4 mg NAA/l. Addition of casein hydrolysate at the rate of 400 mg/l favoured shoot growth.

Dhumale *et al.* (1994) cultured 2-3 mm shoot tips of cultivar 'CoC 671' on modified MS medium containing 3 or 4 mg BAP and 0-2 mg 1AA/l. Per cent induction of multiple shoot and mean multiple shoot number were highest (90 per cent and 16.5, respectively) with 3 mg BAP and 1 mg IAA/l. Hendre *et al.* (1983a) obtained 15-20 shoots from each shoot tip within 2-3 weeks, by elongated shoot tips of 'Co-740', cultured on MS medium supplemented with cytokinins.

The study of Naritoom *et al.* (1993) indicated that shoot initiation from axillary buds of in sugarcane shoot tips cultured in continuously stirred (2 cycles/min) liquid media and incubated in light was the best (4-5 shoots within 20 days) in MS medium supplemented with 0.2 mg BA and.02 mg NAA/l. Shootlets were later multiplied four fold every two weeks by subculturing in the same medium. Further multiplication was carried out on solid MS containing the same growth regulators.

In experiments with cultivar 'Co. Pant 84211' explants, liquid and solid MS media were equally suitable for establishment of explants. But the liquid medium was better for shoot proliferation. Modified MS medium supplemented with 0.5 mg/l of each IAA, BAP and Kinetin was optimum for establishment and 0.1 mg/l IAA + 2mg/l BAP+1mg/l kinetin for proliferation. For establishment and proliferation, 3 per cent sucrose was suitable (Shukla *et al.,* 1994).

2.4.2 Rooting of shoots

Although a number of plants root spontaneously in culture (some monocotyledonous and other herbaceous species such as potato), shoots of most species multiplied *in vitro* lack root system. Rooting of shoots does not occur normally during shoot proliferation due to application of higher doses of cytokinins. In fact it is undesirable to have too many roots at this stage as it might result in wasteful use of energy. As most roots are damaged during subculture, fresh roots have to be initiated again. Besides, the cultures are messy with roots of many shoots. Most protocols prefer clean shoots at this phase, if it could be managed and induce roots in a separate step. Rooting can be achieved either by subculturing on medium lacking cytokinin, with or without an auxin, or by treating the shoots as conventional cuttings after removal from sterile culture. Rooting is also improved in many woody and herbaceous species by lowering the concentration of macro salts to half or less, and the concentration of sucrose from 2 or 3 per cent to 0.5 or 1 per cent. The concentration of rooting hormone required is often critical to provide sufficient stimulus to initiate roots. IBA and NAA are auxins of choice, which stimulate rooting. NAA usually gives rise to short, thick roots.

Mohatkar *et al.* (1993) obtained 4-9 roots of 5-8 cm in length by transferring shoots of 6-7cm height on to half strength of MS liquid medium. Naritoom *et al.* (1993) reported that rooting can be obtained on MS medium with 1 mg / l IBA. Dhumale *et al* .(1994) induced rooting on half strength of MS medium supplemented with 2 mg / l IBA and 1 mg / l IAA. Gildiaz *et al.* (1989) reported that the best root induction and highest survival rate of transplanted plants ware obtained with MS medium supplemented with 7.4 mg / l IAA and 4 per cent sucrose. Most of the workers used normal MS medium with 5 mg NAA / l and varried levels of sucrose; 7 per cent sucrose (Larkin, 1982), 5 per cent sucrose (Shukla *et al* ., 1994) and 6 per cent sucrose (Alam *et al.*, 1995; Islam *et al.*, 1996) to induce roots. Bhansali and Singh (1982) induced rooting with 5 mg / l NAA and 7.5 mg / l IAA. Schenk - Hilderbrandt medium with 5 mg / l NAA was best for rooting (Kharinarain *et al.*, 1996). Santana (1983) reported that 10 mg / l NAA induced strong rooting. Generally root induction in sugarcane is supposed to be less problematic and most shoots root well within 10-15 days on a medium with an auxin.

2.4.3 Hardening and field establishment

Hardening is an important step in micropropagation of most species. Transfer of plantlets from culture vessels to *ex vitro* conditions is a major change in the environment. The initial phase of adjustment is critical. The largest problem concerns the drop in relative humidity from near 100 per cent in the culture vessels to much lower values in the glasshouse or in the field. Excessive water loss usually results from absence of epicuticular wax on *in vitro* shoots (Sutter and Langhans, 1979).

Sreenivasan and Sreenivasan (1992) observed 90 - 95 per cent survival of micropropagated plantlets in glasshouse under shade. They took plantlets with healthy roots, trimmed the leaves and excess roots and transferred to potting medium with a 1:1:1 mixture of sieved sand, silt and ratoon pressed mud.

Shukla *et al.*(1994) observed 100 per cent survival of the plantlets in a hydroponic system and 80 to 90 per cent in potting mixtures of FYM, sand and soil. Satisfactor levels of hardening and establishment of micropropagated plants have been achieved in other studies also (Hendre *et al.*, 1983; Jimenez *et al* ., 1991; Savangikar *et al.*, 1991).

According to the report of Sugarcane Breeding Institute, Coimbatore (Anon., 1993), there was 100 per cent establishment of field planted mericlones compared to that of setts (67 per cent). The tillering capacity was also higher at 6.27 for mericlonal plants versus 1.58 for setts. The yield of mericlone planted crop was more (143 t / ha) compared to sett planted crop (134 t / ha) and there was no difference in juice quality and other parameters. The spacing of 45 cm (intra row) was most economical for mericlone planting.

The cost effectiveness of mericlonal planting was studied by Victoria and Guzman (1993). They cultured the meristem on MS - I, MS - II and MS - III media successively for 30,45 and 45 days, respectively. They obtained 1500 plantlets per meristem. This enables in 4 months, planting of an area that would require 4 years to be planted with traditional propagation method.

Sreenivasan and Sreenivasan (1992) noticed uniform growth, increased vigour and synchronous tillering of the mericlones. They also noticed average increase of yield by 10 t / ha and 0.5 per cent increase in sucrose content. These characters were maintained for another 5 years of vegetative propagation.

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present investigations reported here were conducted at the Tissue Culture Laboratory of the Department of Genetics and plant Breeding, College of Agriculture, Dharwad during 1996-98. The experimental material and the procedures followed are presented here.

3.1 PLANT MATERIAL

The plant material required for the study was collected from Agricultural Research Station, Sankeshwar, University of Agricultural Sciences, Dharwad and Karnataka Institute of Applied Agricultural Research, Sameerawadi. The material consisted of three popular cultivars of sugarcane: *viz.*,

'Co 8014'

'Co 85002'

'CoC 671'

3.2 EXPERIMENTAL CONDITIONS

All the laboratory experiments were conducted under well defined conditions of the culture room maintained at 25 $\pm$ 2°C. Uniform light (Ca.1000 lux) was provided by fluorescent tubes (7200 °K) over a light / dark cycle of 16 / 8 hours.

Culture work was carried out aseptically in a laminar air flow chamber. For all the experiments, borosilicate glasswares and analytical grade chemicals were used. The explants were cultured in 150 X 25 mm tubes containing 12 ml of appropriate medium.

3.3 NUTRIENT MEDIUM

Murashige and Skoog's (1962) medium (MS) commonly used by several workers for sugarcane tissue culture was made use of in this study. This medium was supplemented with different growth regulators at varying concentrations, depending upon the purpose of the individual experiment. The composition of the medium used is given bellow:

Composition of MS medium

	Components	**mgl^{-1}**
Macronutrients	$NH_4 NO_3$	16,50.000
	KNO_3	1,900.000
	$MgSO_4. 7H_2O$	370.000
	$NaH_2 PO_4$	170.000
	$CaCl_2.2H_2O$	440.000
	$FeSO_4.7H_2O$	27.800
	Na_2. EDTA	37.800
Micronutrients - I	KI	0.830
	$H_3 BO_3$	6.300
	$MnSO_4.7H_2O$	22.300
	$ZnSO_4.7H_2O$	8.600
	$Na_2 MoO_4.2H_2O$	0.250
Micronutrients - II	$CuSO_4. 5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Organics	Inositol	100.000
	Thiamine HCl	2.000
	Pyridoxine HCl	0.500
	Glycine	10.000
Energy source	Sucrose	30,000.000
Solidifying agent	Agar	9,000.000

3.4 PREPARATION OF MEDIUM

Medium was composed from separate stocks (20 x) of macronutrients, micronutrients, Fe - EDTA and organics stored in the refrigerator (7 - 8°C). Inositol, sucrose and agar were weighed and added in required amounts at the time of media preparation. Growth regulators were added from stock solutions of suitable strength. After mixing all the ingredients one by one, including sucrose (except agar), double distilled water was added to make it up to just short of the final volume. The pH of the medium was adjusted to 5.6 - 5.8 by using either 0.1N HCl or NaOH, with the help of digital pH meter. Volume was finally adjusted and required amount of agar was added. The medium containing agar was heated gently to 90 - 95°C until agar melted completely. The hot medium was then dispensed in suitable containers, closed with either non - absorbant cotton or heat resistant polythene caps and autoclaved at 1.0kg / sq cm (121°C) for 15min. After sterilisation by autoclaving the medium was allowed to solidify at room temperature and stored at least for two days before use.

3.5 PREPARATION OF EXPLANTS

Young and healthy shoots (tops) from field grown plants of different clones were collected to obtain explants. The shoots were trimmed by removing older leaves and visible internodes, successively, to get 10 -15 cm long columns (Platela) having tightly held leaf sheaths, enclosing the apical meristem, young leaves and leaf sheaths. These columns were individually surface sterilised by dipping in 70 per cent ethyl alcohol for one minute and then air dried for a couple of minutes to remove excess alcohol.Further the outer leaf sheaths were slowly and carefully removed aseptically, without injuring the apical meristem. After removing the leaf sheaths, the apical meristem of about 1 - 2 mm size were inoculated into culture tubes individually. The meristems thus obtained had meristem dome and visible leaf primordium.

3.6 EXPERIMENTAL DETAILS

3.6.1 Initiation of shoot tip cultures

The apical meristems of all three cultivars of sugarcane were established on MS medium containing various levels of benzyladenine (BA) following usual aseptic procedures. These were subcultured on the respective medium after 25 days of incubation.

The meristems normally grew into a single shoot. Such shoots were allowed to grow further and produce multiple shoots. The shoots so obtained were transferred to MS medium containing various combinations and concentrations of growth regulators, *viz.*, BA and NAA. Observations on the shoot proliferation rate, number and nature of shoots were recorded 25 days after inoculation. The multiple shoot cultures were routinely maintained by subculturing a bunch of 2 - 3 shoots after every 25 days.

3.6.2 Use of different sources and levels of sucrose

Normally analytical grade sucrose was used for tissue culture studies. Different levels of sucrose (AR) were tried against the control (30 gl^{-1}) to study their effect on shoot multiplication and quality of shoots obtained. In order to reduce the cost of culture medium, sucrose of different grades were used at a level determined as optimum by previous experiments. The type of sucrose used were analytical grade

(AR), laboratory grade (LR) obtained from sd Fine Chemicals, commercially available diamond sugar and ordinary tea sugar (commercial sugar).

3.6.3 Use of different supporting agents

Agar (9 g / l) was routinely used as solidifying agent in MS medium. In order to reduce the cost of medium, alternative supporting agents, *viz*., peat, sand and sabudani (sago) were tried.

Peat and sand (Passed through a sieve of 2 x 2 mm pore size) usually contains minerals in small quantities. So they were separately soaked in 0.1N HCl for 24 hours and then washed with running water to remove the acid completely and then rinced in distilled water. These materials were separately filled into the test tubes to required levels and MS medium (excluding agar) was added up to the level of peat / sand.

Commercially available sabudani (Sago) in the form of white, hard pearls was ground to fine flour and different amount of this powder was mixed in suitable volume of water. The mixture was boiled until sabudani melted and allowed to cool at room temperature. The optimum concentration of sabudani which gave required degree of gelling was selected. The concentration of 100 gl^{-1} gave optimum degree of gelling and it was used in MS medium in place of agar.

3.6.4 Root induction and establishment of plant

The multiple shoots obtained from shoot proliferation medium were separated and individual shoots were transferred to MS medium supplemented with different levels of NAA for rooting. The MS medium supplemented with 1 gl^{-1}

activated charcoal was also used as rooting medium. The shoots were allowed to grow in the test tubes to a height of about 8 - 10 cm. Plentlets with 4 - 5 healthy roots were transplanted in polythene bags (18 x 12 cm) containing sterilised potting medium (biogas slurry, soil and sand in 2: 1: 1 proportion). The plants were raised in glasshouse for establishment and further growth for a period of two months.

3.7 STATISTICAL PROCEDURES AND ANALYSIS OF DATA

As all the studies were done in the laboratory under well defined conditions of medium of growth, temperature and light, completely randomised design (CRD) was employed for the experiments.

The observations from individual genotypes were analysed using simple CRD. Data from different genotypes obtained form similar experiments were pooled. The pooled data was analysed as a factorial experiment. For analysis of the data standard statistical procedures as outlined by Cochran and Cox (1957) were followed.

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Laboratory experiments were conducted during 1997 - 98 to study different parameters of micropropagation in popular caltivars of sugarcane, *viz*., 'Co 8014', 'Co 85002' and 'CoC 671'. The results of these experiments are presented under the following heads.

4.1 Initiation and establishment of meristem culture.

4.2 Shoot multiplication.

4.3 Rooting of shoots.

4.4 Establishment of plantlets.

4.1 INITIATION AND ESTABLISHMENT OF MERISTEM CULTURE

The apical meristems of three cultivars ('Co 8014', 'Co 85002' and 'CoC 671') were inoculated with different concentrations of BA for shoot initiation. Small shoots started appearing within 6 -7 days in many of the culture tubes. Initially there was problem of tissue browning, which slightly hindered the shoot growth. Subculturing was done frequently (10 -15 days) during the early stages of establishment to reduce the adverse effect of browning. After 3 - 4 subcultures there was drastic reduction in browning of tissues and the release of pigments in the medium.

The per cent establishment varied between different cultivars and levels of BA. In the cultivar 'Co 8014', the frequency of establishment was the highest (83 per cent) on MS medium with 2.0 mgl^{-1} BA followed by 80 per cent with 1.0 mgl^{-1} BA and the lowest (40 per cent) was with 0.5 mgl^{-1} BA.

Likewise, the cultivar 'Co 85002' recorded the highest response (80 per cent) with 2 mgl^{-1} BA and the lowest was with 0.5 mgl^{-1} BA (8 per cent). The cultivar 'CoC 671' resulted in maximum frequency of establishment (72 per cent) with 2.0 mgl^{-1} BA and minimum (8 per cent) was with 0.5 mgl^{-1} BA (Table 1).

Frequency of establishment was maximum with 2.0 mgl^{-1} BA across all the cultivars, on which the shoots were active and healthy compared to other treatments. At this level of BA, cultivar 'Co 8014' was the most responsive followed by 'Co 85002' and 'CoC 671'. About 10 - 15 shoots originated from each single meristem by the end of 25 days.

4.2 SHOOT MULTIPLICATION

4.2.1 Effect of BA and NAA on shoot multiplication

Once the meristems were established and sufficient number of shoots were available, an experiment involving various concentrations and combinations of growth regulators, *viz*., BA (0. 5, 1.0 and 2.0 mgl^{-1}) and BA (2 mgl^{-1}) + NAA (1 mgl^{-1}) was conducted to study their effect on shoot multiplication. The rate of multiplication of different cultivars with different concentrations of growth regulators is presented in Table 2 and depicted in Fig. 1.

The cultivars 'Co 8014' showed great variation for mean rate of shoot multiplication with different concentrations of growth regulators (Table 2a). The mean rate of multiplication was the highest (20.05) with 2.0 mgl^{-1} BA and was significantly superior to all other concentrations. But the shoots were in bunches and individual shoots were tiny, weak and non separable. Considering the further

Table 1: Effect of BA on establishment of meristem cultures of 'Co 8014', 'Co 85002' and 'CoC 671'

Sl. No.	Genotype	BA concentration (mgl^{-1})	No. of shoot tips inoculated	No.of shoot tips established	Per cent establishment
1	Co 8014	0.5	25	10	40
2		1.0	25	20	80
3		2.0	30	25	83
4	Co 85002	0.5	25	2	8
5		1.0	30	12	40
6		2.0	30	24	80
7	CoC 671	0.5	25	2	8
8		1.0	25	5	20
9		2.0	25	18	72

requirements of culture, such shoots were found undesirable. On MS medium with 1.0 mgl^{-1} BA (Plate 1b) the shoots were well formed, thick and easily separable from one another. This treatment could support a multiplication ratio of 7.67, which was superior to all the other treatments except the one with 2.0 mgl^{-1} BA. The rate of multiplication with 0.5 mgl^{-1} BA and 2 mgl^{-1} BA + 1 mgl^{-1} NAA was 6.22 and 3.27, respectively (Plate 1a & 1c).

The response in 'Co 85002' was similar to that of 'Co 8014' (Table 2b). The mean rate of multiplication was 20.00 at 2.0 mgl^{-1} BA and was significantly superior compared to all other treatments. But, the shoots were of inferior quality. The quality of shoots on 1mgl^{-1} BA was better (Plate 2b). The mean rate of multiplication was also high (6.05) and significantly superior to other treatments, except that at 2.0 mgl^{-1} BA. Treatment with 0.5 mgl^{-1} BA recorded moderate (4.77) rate of shoot multiplication but the shoots were of better quality (Plate 2a). Minimum rate of multiplication observed was 3.60 at BA(2.0 mgl^{-1}) + NAA (1.0 mgl^{-1}) and the plantlets were tiny and weak (Plate 2c).

The cultivar 'CoC 671' recorded the highest rate of multiplication (19.83) at 2.0 mgl^{-1} BA and the least was 2.11 with BA(2.0) + NAA(1.0). Both of these treatments resulted in poor quality plantlets (Plate 3b). The mean rate of multiplication was 5.05 at 1.0 mgl^{-1} BA and was significantly superior to other treatments except the one with 2.0 mgl^{-1} BA. The plantlets were of desirable quality (Plate 3c). Though the shoots at 0.5 mgl^{-1} BA were well formed, the rate multiplication was only 2.27 (Table 2c).

Table 2 Effect of BA and NAA on shoot multiplication in different cultivars of sugarcane

a: 'Co 8014'

Sl. No.	Growth regulator	Concentration (mgl^{-1})	** Average No. of shoots (25 DAI)
1	BA	0.5	6.22
2		1.0	7.67
3		2.0	20.05
4	BA + NAA	2.0+1.0	3.27
SEm			0.35

b : 'Co 85002'

Sl. No.	Growth regulator	Concentration (mgl^{-1})	** Average No. of shoots (25 DAI)
1	BA	0.5	4.77
2		1.0	6.05
3		2.0	20.00
4	BA + NAA	2.0+1.0	3.60
SEm			0.87

c: 'CoC 671'

Sl. No.	Growth regulator	Concentration (mgl^{-1})	** Average No. of shoots (25 DAI)
1	BA	0.5	2.27
2		1.0	5.05
3		2.0	19.83
4	BA + NAA	2.0+1.0	2.11
SEm			0.36

** Significant at P= 0.01

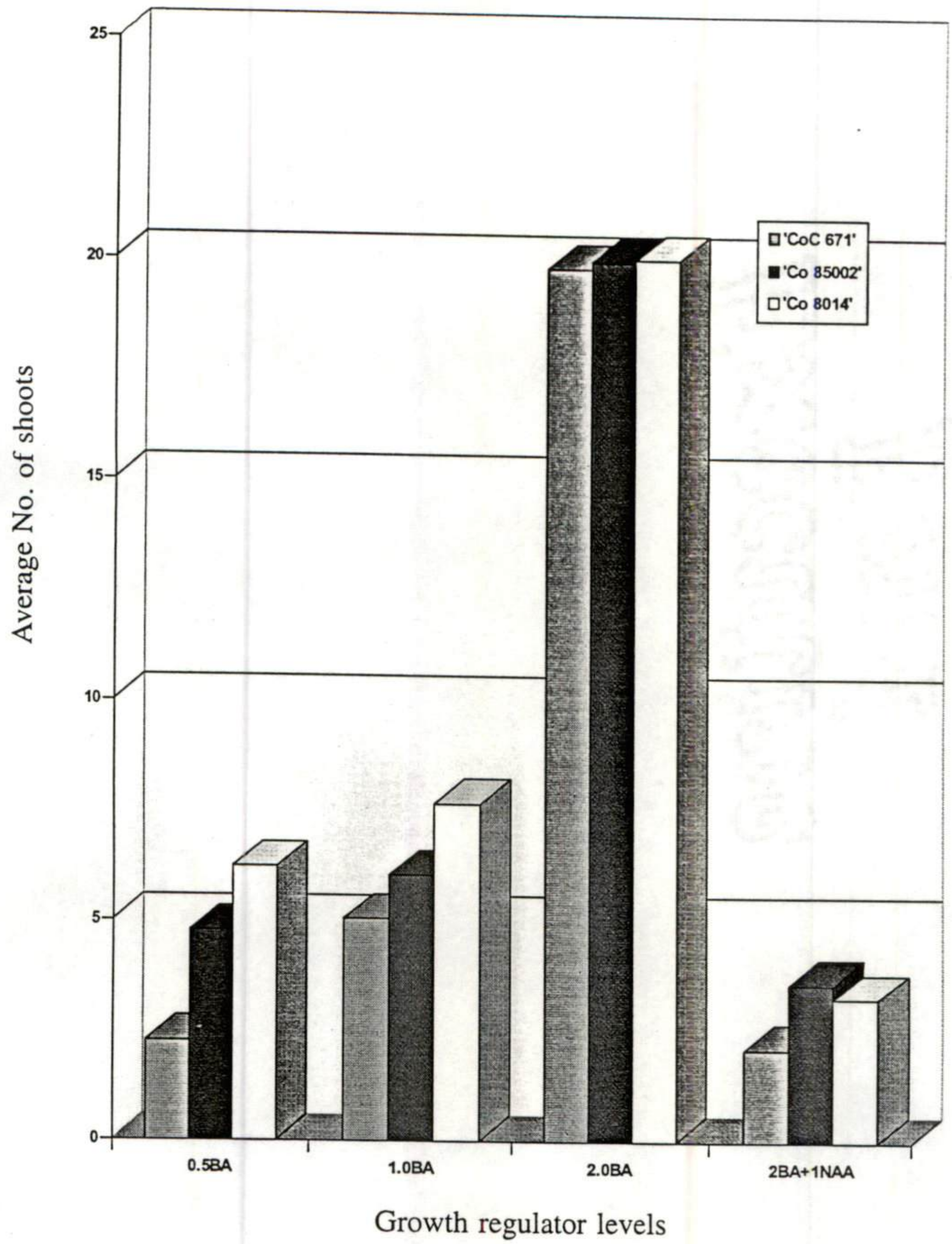

Fig.1 : Effect of BA and NAA on shoot multiplication in different cultivars of sugarcane

Plate 1: Photograph showing shoot multiplication in ‘Co 8014’ with BA and NAA

a. 0.5 mgl^{-1} BA
b. 1.0 mgl^{-1} BA
c. 2 mgl^{-1} BA + 1 mgl^{-1} NAA

Plate 2: Photograph showing shoot multiplication in ‘Co 85002’ with BA and NAA.

a. 0.5 mgl^{-1} BA
b. 1.0 mgl^{-1} BA
c. 2 mgl^{-1} BA + 1 mgl^{-1} NAA

Plate 3: Photograph showing shoot multiplication in ‘CoC 671’ with BA and NAA.

a. 0.5 mgl^{-1} BA
b. 1.0 mgl^{-1} BA + 1 mgl^{-1} NAA
c. 1.0 mgl^{-1} BA

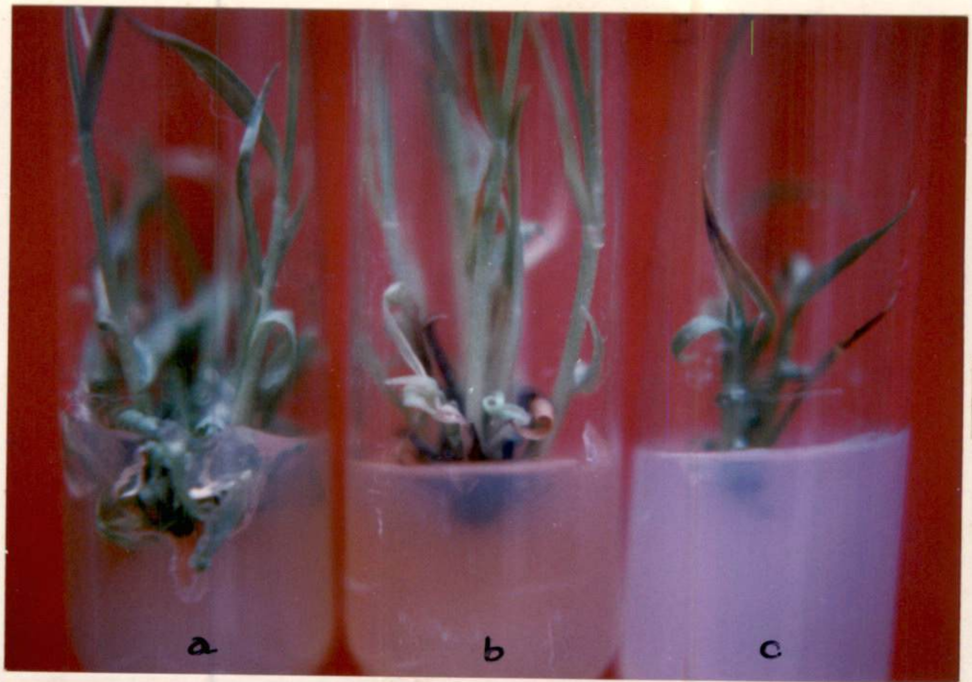

Plate 1

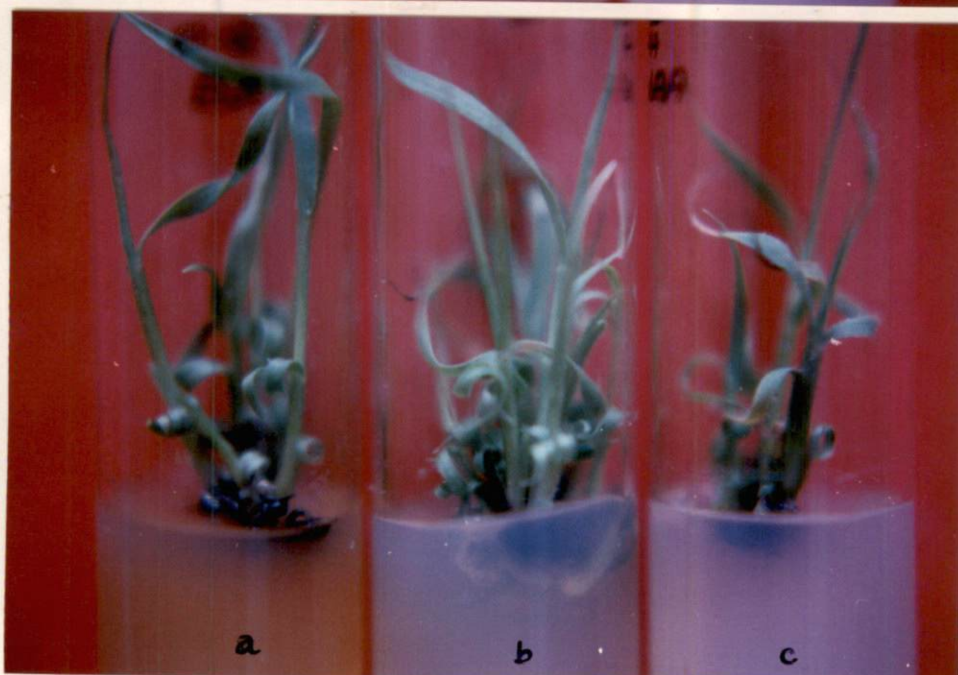

Plate 2

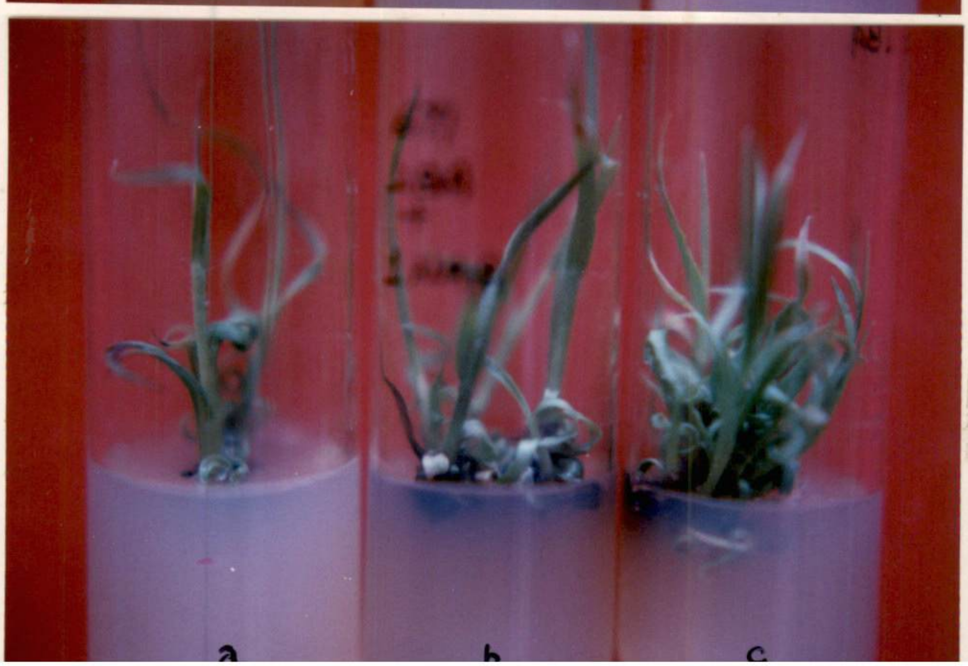

Plate 3

The data obtained individually on the three cultivars, from four treatments, with respect to shoot multiplication were pooled and analysed as a factorial experiment to know the interaction between the factors.The ANOVA revealed significant differences in response due to genotypes and growth regulator levels (Table 3). The interaction component was also statistically significant.

Among the genotypes, 'Co 8014' recorded the highest overall rate of shoot multiplication (9.31), followed by 'Co 85002' (8.71) and 'CoC 671' (7.33). The differences among the cultivars were statistically significant. In case of the treatments, 2.0 mgl^{-1} BA recorded the highest overall multiplication rate(20.09) followed by 1.0 mgl^{-1} BA(6.26) and 0.5 mgl^{-1} (4.43) and the least (3.00) was at BA (2 mgl^{-1}) + NAA(1 mgl^{-1}) (Table 4).

4.2.2 Effect of BA during II cycle of shoot multiplication

Two best treatments (1.0 mgl^{-1}BA and 0.5 mgl^{-1} BA) were selected based on the mean rate of multiplication and the quality of plantlets, to study their effect during II cycle of shoot multiplication (Table 5). Both these treatments resulted in high rate of multiplication and the plantlets obtained were of desirable quality, across the genotypes.

In cultivar 'Co 8014', the rate of multiplication (6.27) at 1.0 mgl^{-1} BA was significantly superior (Table 5a). But this was less compared to multiplication rate during I cycle (7.67) at the same concentration of BA. The rate of multiplication at 0.5 mgl^{-1} (4.66) was also lower than that during I cycle (6.22). There was no difference in the quality of the shoots obtained in both the cases.

Table 3: ANOVA for shoot multiplication in different genotypes of sugarcane with BA and NAA

Source of variation	DF	SS	MSS	F
Genotype	2	149.52	74.76	35.54**
Growth regulator levels	3	10057.22	3352.40	1593.73**
Genotype X Growth regulator levels	6	81.472	13.57	6.45**
Error	204	429.11	2.103	

** Significant at P=0.01

Table 4: Comparison of the effect of genotype and the levels of BA and NAA on shoot multiplication

Genotype	Mean (No. of shoots)	Growth regulator (mgl^{-1})	Mean (No. of shoots)
Co 8014	9.31	BA 0.5	4.43
Co 85002	8.71	1.0	6.26
CoC 671	7.33	2.0	20.09
		BA (2.0) + NAA (1.0)	3.00
SEm	0.17		0.20
CD (at 5 per cent)	0.47		0.55

Similar response was observed in cultivar 'Co 85002' (Table 5b). Here the highest rate of multiplication was 4.91 with 1.0 mgl^{-1} BA and was significantly better than the other treatment. The lowest multiplication rate was 2.86 at 0.5 mgl^{-1} BA. However, there was reduction in mean rate of multiplication compared to I cycle of shoot multiplication (6.05 and 4.77 at 1.0 mgl^{-1} BA and 0.5 mgl^{-1} BA respectively). The quality of the shoots was similar in both the cycles of multiplication.

Likewise, in 'CoC 671' the mean rate of multiplication was 4.61 with 1.0 mgl^{-1} BA and 2.58 with 0.5 mgl^{-1} BA. The difference in the rate of multiplication was statistically significant. There was a reduction in the response from I cycle (5.05) to II cycle (4.61). But there was a slight increase in multiplication rate (2.58) during II cycle (Table 5c).

The data obtained from similar experiments on individual culitvars were pooled and analysed as a factorial experiment to ascertain interaction effects (Table 6). The ANOVA shows high significant differences between the treatments as well as genotypes. But the interaction component was not significant.

The highest mean rate of multiplication (5.47) across all treatments was observed in cultivar 'Co 8014', followed by 'Co 85002' (3.89) and 'CoC 67' (3.60). Of the two treatments 1.0 mgl^{-1} BA was better (5.27) than at 0.5mgl^{-1} BA (3.27) (Table 7).

4.2.3 Effect of different sources of sucrose on shoot multiplication

Usually analytical grade sucrose (AR) is used in laboratory experiments as a carbon source. An attempt was made to replace the analytical grade

Table 5: Effect of BA levels on different cultivars of sugarcane during II cycle of shoot multiplication

a: 'Co 8014'

Sl. No.	BA level (mgl^{-1})	** Average No. of shoots (25 DAI)
1	0.5	4.66
2	1.0	6.27
SEm		0.16

b: 'Co 85002'

Sl. No.	BA level (mgl^{-1})	** Average No. of shoots (25 DAI)
1	0.5	2.86
2	1.0	4.91
SEm		0.17

c: 'CoC 671'

Sl. No.	BA level (mgl^{-1})	** Average No. of shoots (25 DAI)
1	0.5	2.58
2	1.0	4.61
SEm		0.16

** Significant at P=0.01

Table 6: ANOVA for shoot multiplication during II cycle in different genotypes of sugarcane with BA

Source of variation	DF	SS	MSS	F
Genotypes	2	73.29	36.64	77.80**
BA levels	1	97.28	97.28	206.54**
Genotypes X BA levels	2	1.11	0.55	1.18
Error	102	48.04	0.47	

** Significant at P=0.01

Table 7: Comparison of the effect of genotypes and the levels of BA on shoot multiplication during II cycle

Genotype	Mean (No. of shoots)	BA levels	Mean (No. of shoots)
Co 8014	5.47	0.50	3.37
Co 85002	3.89	1.00	5.27
CoC 671	3.60		
SEm	0.11		0.09
CD (at 5 per cent)	0.30		0.25

sucrose by different sources of sucrose, *viz.*, laboratory grade sucrose (LR), diamond sugar and ordinary sugar. An experiment was conducted to assess the effect of these sources of sucrose (all at 30 gl^{-1}) in comparison to analytical grade sugar (30 gl^{-1}). The response is shown in Fig. 2.

The response of cultivar 'Co 8014' to different sources of sucrose is presented in Table 8a. The highest mean rate of multiplication (6.02) was recorded with the control (AR). But it was statistically on par with all other treatments (Plate 4). The lowest multiplication rate was 5.72 with the treatment, where commercial grade sugar was used. The mean rate of multiplication was 5.91 with laboratory grade sucrose (LR) and 5.86 with diamond sugar.

Similar results were recorded with genotype 'Co 85002' where analytical grade sucrose gave maximum response compared to all other treatments. But there was no significant difference between the treatments (Plate 5). The mean rate of multiplication was the highest (5.58) with control (AR) and the least (5.11) was observed with commercial grade sugar. The multiplication rate observed with LR and diamond sugar were 5.30 and 5.22 respectively (Table 8b).

In cultivar 'CoC 671' also the responses observed in different treatments were statistically significant (plate 6). The maximum rate of multiplication (5.16) was in analytical grade sugar followed by laboratory grade sugar (5.19). Both the treatments with diamond sugar and commercial grade sugar resulted in the low (4.88) multiplication rate, comparatively (Table 8c).

The data obtained on individual cutivars from different treatments with respect to rate of multiplication were pooled and analysed as factorial experiments.

Table 8: Effect of different sources of sucrose on shoot multiplication in different cultivars of sugarcane

a: 'Co 8014'

Sl. No.	Sources of sucrose	(NS) Average No. of shoots (25 DAI)
1	Analytical grade (AR) (control)	6.02
2	Laboratory grade (LR)	5.91
3	Diamond sugar	5.86
4	Ordinary sugar	5.72
SEm		0.18

b: 'Co 85002'

Sl. No.	Sources of sucrose	(NS) Average No. of shoots (25 DAI)
1	Analytical grade (AR) (control)	5.58
2	Laboratory grade (LR)	5.30
3	Diamond sugar	5.22
4	Ordinary sugar	5.11
SEm		0.17

c: 'CoC 671'

Sl. No.	Sources of sucrose	(NS) Average No. of shoots (25 DAI)
1	Analytical grade (AR) (control)	5.16
2	Laboratory grade (LR)	5.19
3	Diamond sugar	4.88
4	Ordinary sugar	4.88
SEm		0.15

NS: Non significant

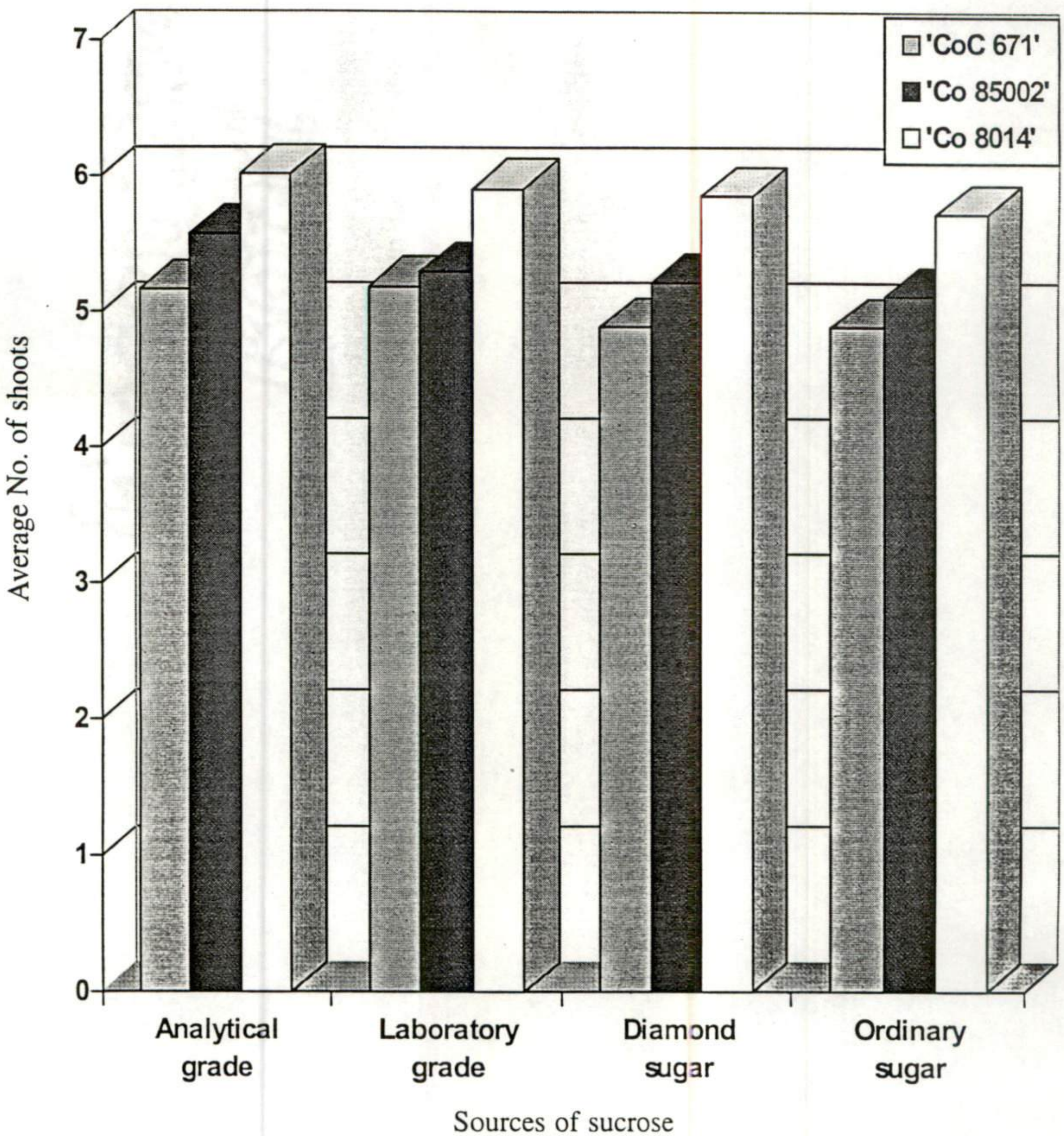

Fig.2 : Effect of different sources of sucrose on shoot multiplication in different cultivars of sugarcane

Plate 4: Effect of different sources of sucrose on shoot multiplication in 'Co 8014'

a. Analytical grade
b. Laboratory grade
c. Diamond sugar
d. Ordinary sugar

Plate 5: Effect of different sources of sucrose on shoot multiplication in 'Co 85002'

a. Analytical grade
b. Laboratory grade
c. Diamond sugar
d. Ordinary sugar

Plate 6: Effect of different sources of sucrose on shoot multiplication in 'Co 671'

a. Analytical grade
b. Diamond sugar
c. Ordinary sugar

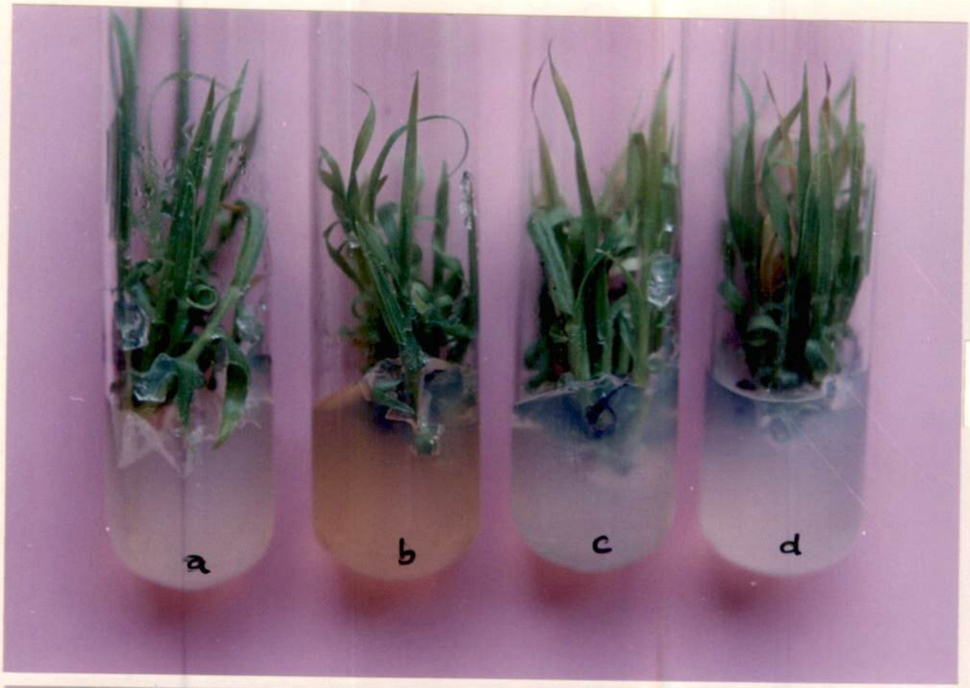

Plate 4

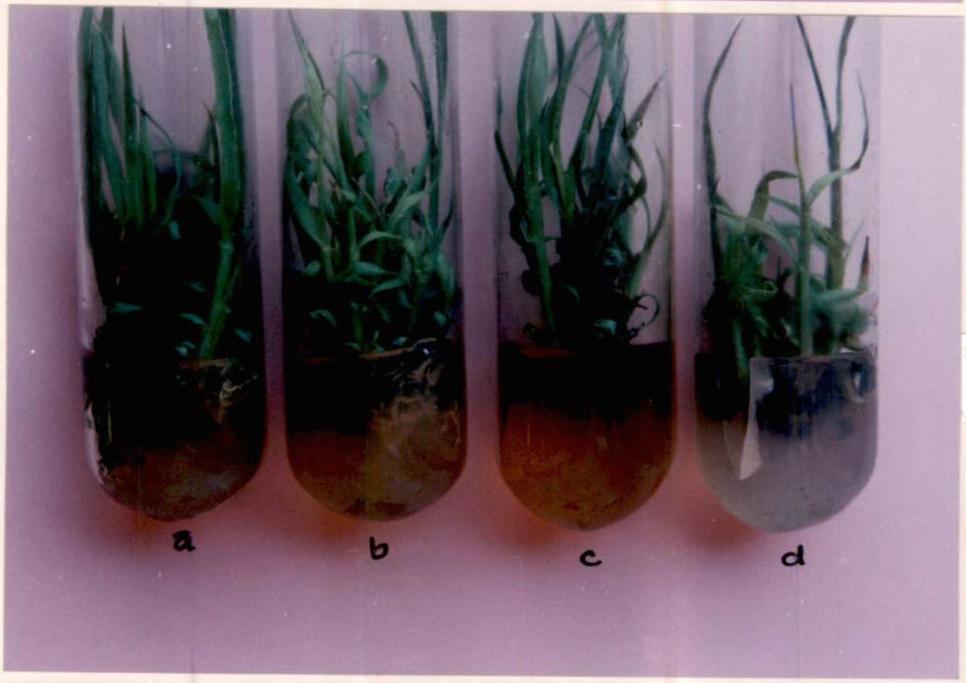

Plate 5

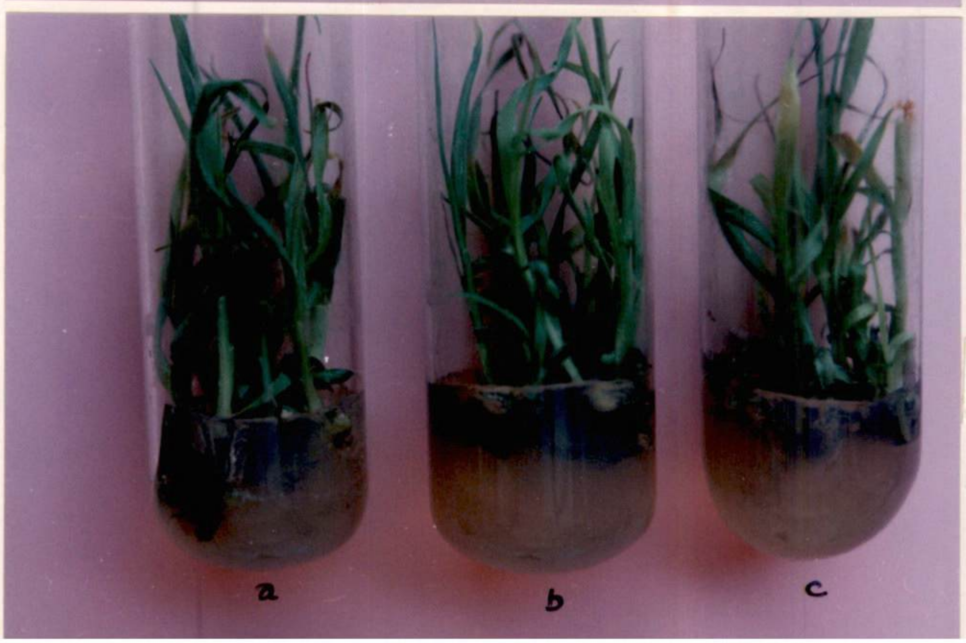

Plate 6

The ANOVA indicates significant differences between the genotypes, but not due to different source of sucrose. The interaction component was also statistically non significant (Table 9).

Among the cultivars, 'Co 8014' resulted in the highest overall rate of multiplication (5.88), which was significantly higher than in 'Co 85002' (5.31) and 'CoC 671' (5.01).

Of the four treatments, overall rate of shoot multiplication was maximum (5.51) with the control (AR). Other treatments, *viz.*, LR grade sucrose, diamond sugar and ordinary sugar recorded 5.47, 5.32 and 5.24 mean rate of shoot multiplication across all the cultivars, respectively. The response observed due to all four treatments were on par with each other (Table 10).

4.2.4 Effect of sucrose levels on shoot multiplication

An experiment was set up to study the effect of different concentrations of sucrose (AR grade) on shoot multiplication rate and nature (quality) of plantlets. Different concentrations of sucrose used were 15 gl^{-1}, 30 gl^{-1} (Control), 45 gl^{-1} and 60 gl^{-1}. The effect of sucrose levels on cultivars of sugarcane is depicted in Fig. 3.

There were differences in the response of 'Co 8014' with respect to mean rate of shoot multiplication and nature of plantlets at different levels of sucrose (Table 11a). The highest mean rate of multiplication was 5.57 with the control (30 gl^{-1}) and it was significantly superior compared to all other treatments. The shoots were strong, healthy and easily separable from one another (Plate 7a). The rate of shoot

Table 9: ANOVA for shoot multiplication in different genotypes of sugarcane with different sources of sucrose

Source of variation	DF	SS	MSS	F
Genotypes	2	29.96	13.48	26.80**
Source of sucrose	3	3.95	1.31	2.62NS
Genotypes X Source of sucrose	6	102.58	0.50	0.21NS
Error	204	102.58	0.50	

** Significant at P = 0.01

NS: Non significant.

Table 10: Comparison of the effect of genotype and sources of sucrose on shoot multiplication.

Genotype	Mean (No. of shoots)	Sources of Sucrose	Mean (No. of shoots)
Co 8014	5.88	AR grade (control)	5.51
Co 85002	5.31	LR grade	5.47
CoC 671	5.03	Diamond sugar	5.32
		Ordinary sugar	5.24
SEm	0.08		0.10
CD (at 5 per cent)	0.22		0.28

multiplication was the lowest (1.55) at 60 gl^{-1}. Further, the plantlets obtained were of poor quality. They were short, thick, slow growing and appeared to experience stress (Plate 7d). The medium with 15 gl^{-1} also resulted in poor quality of plantlets, i.e. the plantlets were very thin and weak (Plate 7b). The mean rate of multiplication observed in this treatment was 3.0. The treatment, 45 gl^{-1} resulted in shoots of better quality, although the multiplication rate was low (1.95).

In the cultivar 'Co 85002', mean rate of shoot multiplication was maximum (5.30) at 30gl^{-1} and the shoots were of desirable quality (Plate 8b). The lowest rate of multiplication (1.25) was with 60 gl^{-1}. Here the shoots were of poor quality (Plate 8d).Better quality plantlets were obtained with 45 gl^{-1}(Plate 8c). In this treatment multiplication rate (3.90) was low compared to 30 gl^{-1}. Sucrose at 15 gl^{-1} resulted in inferior quality plantlets (Plate 8a) and the rate of multiplication recorded was 3.45 (Table11b).

The response of 'CoC 671' to different levels of sucrose was similar to that of 'Co 8014' (Table 11c). The highest mean rate of multiplication (5.15) was observed with the control (30 gl^{-1}). Here the shoots were of better quality compared to other treatments (Plate 9b). This treatment was also statistically superior to the all other treatments with respect to mean rate of shoot multiplication. Treatment with 45 gl^{-1} sucrose recorded a mean rate of multiplication of 2.95 and the quality of shoots was better than at either 60 gl^{-1} or 15 gl^{-1} (Plate 9d). Poor quality shoots were observed at 15 gl^{-1} with low mean rate (2.5) of multiplication (Plate 9a) and it was the lowest (1.5) with 60gl^{-1} and the plantlets were of inferior quality (Plate 9c).

Table 11: Effect of sucrose levels on shoot multiplication in different cultivars of sugarcane

a: 'Co 8014'

Sl. No.	Sucrose (gl^{-1})	** Average No. of shoots (25 DAI)
1	15	3.00
2	30	5.57
3	45	1.95
4	60	1.55
SEm		0.17

b: 'Co 85002'

Sl. No.	Sucrose (gl^{-1})	** Average No. of shoots (25 DAI)
1	15	3.45
2	30	5.30
3	45	3.90
4	60	1.25
SEm		0.22

c: 'CoC 671'

Sl. No.	Sucrose (gl^{-1})	** Average No. of shoots (25 DAI)
1	15	2.50
2	30	5.15
3	45	2.95
4	60	1.50
SEm		0.20

** Significant at P = 0.01

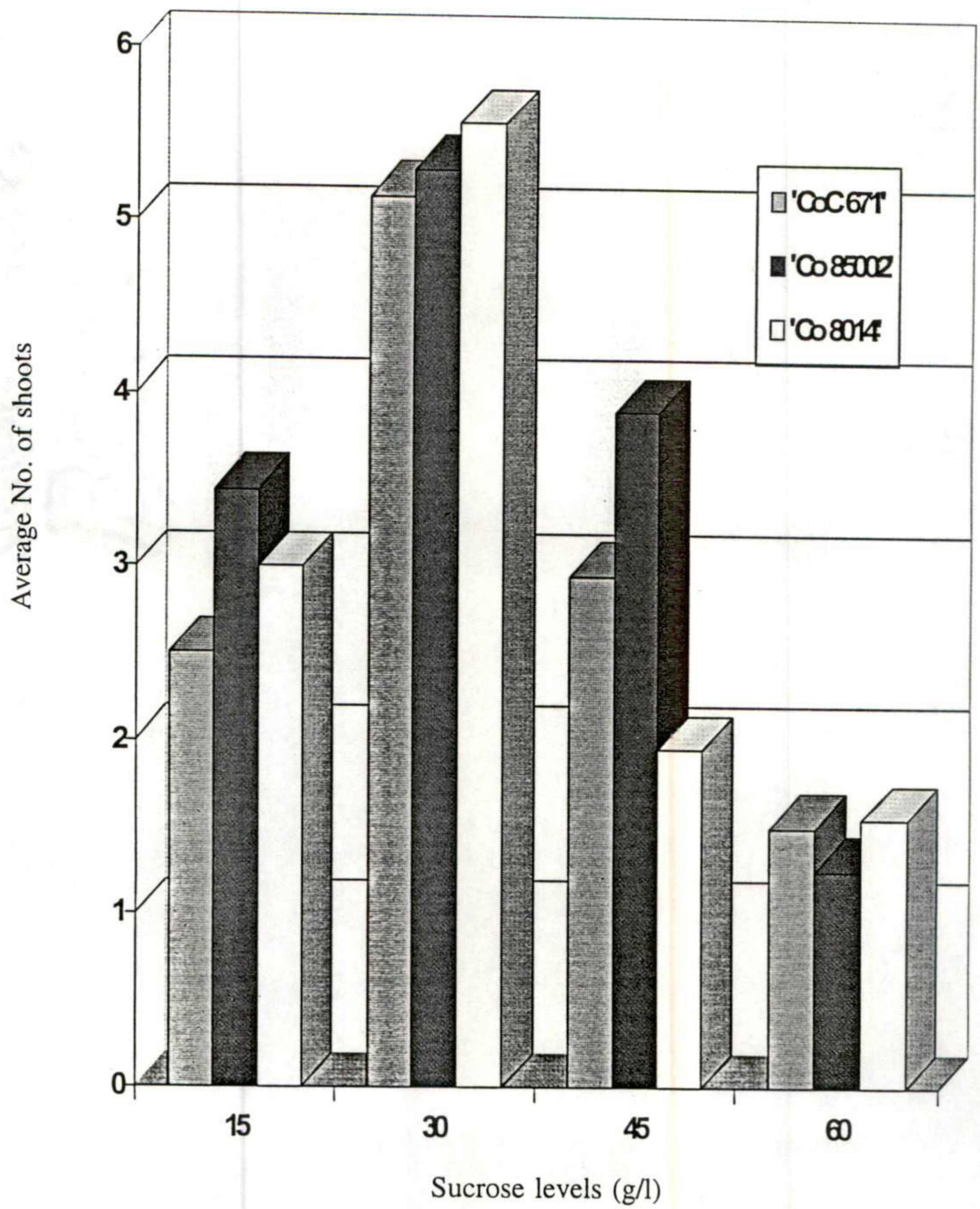

Fig.3 : Effect of different levels of sucrose on shoot multiplication in different cultivars of sugarcane

Plate 7: Shoot multiplication in 'Co 8014' with different levels of sucrose

a. 30 gl^{-1}
b. 15 gl^{-1}
c. 45 gl^{-1}
d. 60 gl^{-1}

Plate 8: Shoot multiplication in 'Co 85002' with different levels of sucrose

a. 15 gl^{-1}
b. 30 gl^{-1}
c. 45 gl^{-1}
d. 60 gl^{-1}

Plate 9: Shoot multiplication in 'Co 671' with different levels of sucrose

a. 15 gl^{-1}
b. 30 gl^{-1}
c. 45 gl^{-1}
d. 60 gl^{-1}

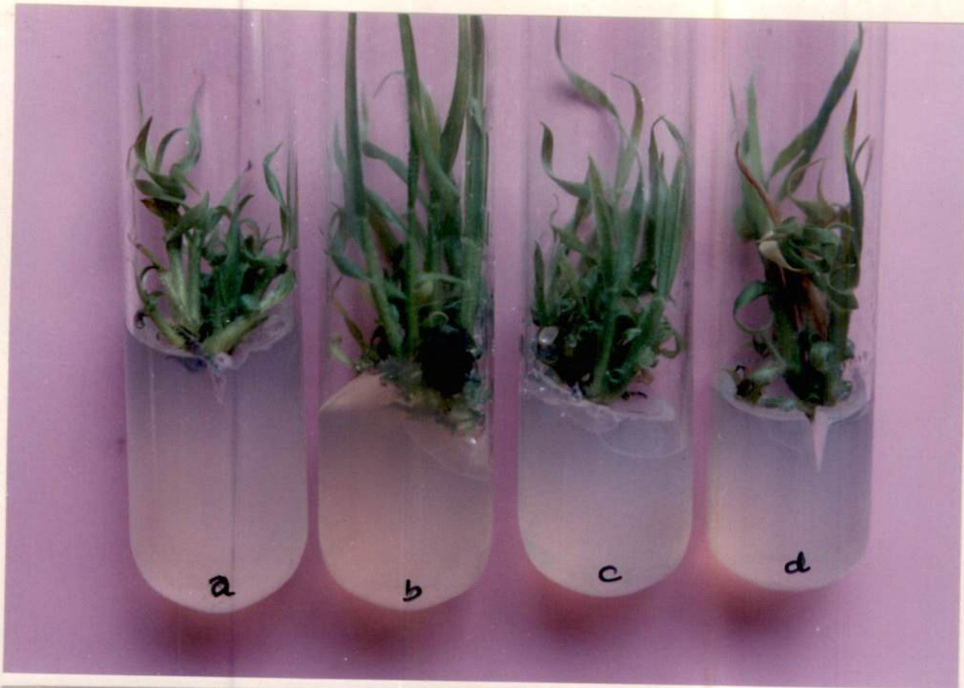

Plate 7

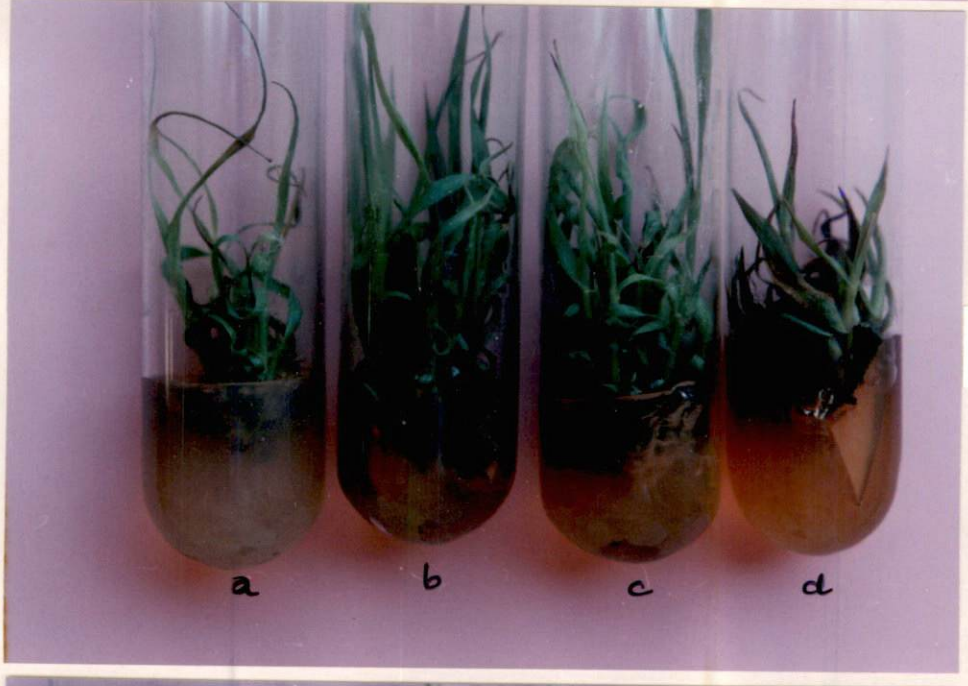

Plate 8

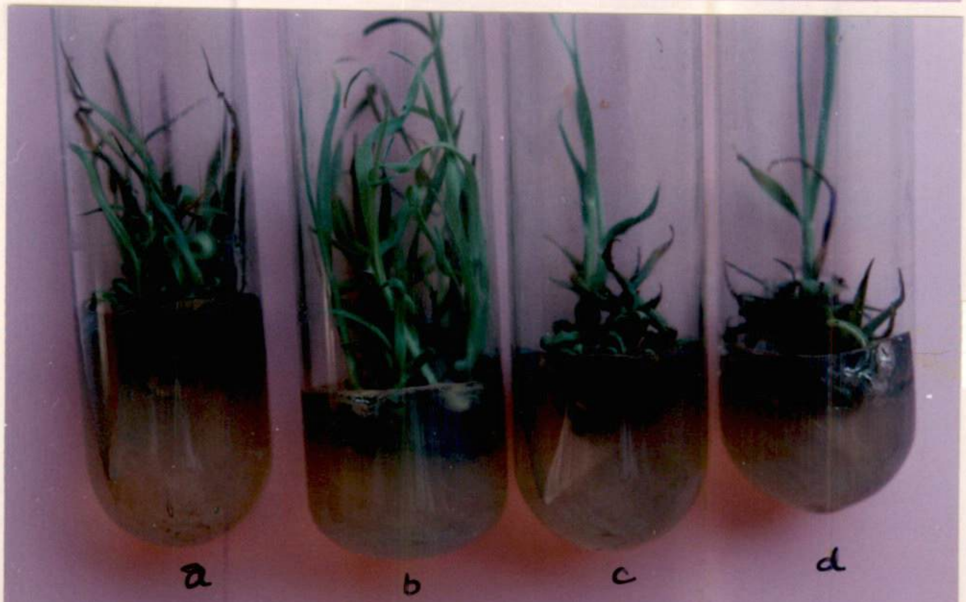

Plate 9

To know the interaction effects due to genotypes and sucrose levels, the data obtained on individual cultivars from similar experiments with respect to rate of multiplication were pooled and analysed as a factorial experiment (Table 12). There was significant difference in the response obtained with respect to rate of multiplication at the genotype and the levels of sucrose. Interaction between genotype and levels of sucrose was also statistically significant.

The cultivar 'Co 85002' recorded the highest overall mean rate of multiplication (3.48), followed by 'Co 8014' (3.08).The rate of shoot multiplication at 30gl^{-1} was the highest (5.57) in cultivar 'Co 8014' compared to other cultivars (Table 11). The mean rate of multiplication across sucrose levels was the lowest (2.99) in the cultivar 'CoC 671' (Table 13).

Out of the sucrose levels, the multiplication rate was maximum (5.40) across all genotypes with the control (30 gl^{-1}) compared to other treatments. The lowest overall multiplication rate recorded was 1.38 at 60 gl^{-1}. The treatments, 15 gl^{-1} and 45 gl^{-1} recorded 3.0 and 2.93 shoots per culture, respectively (Table 13).

4.2.5 Effect of different supporting agents on shoot multiplication ugarcane

It is a standard procedure to use gelled agar as a physical support for the *in vitro* cultures. Alternative sources of supporting agents, *viz*., acid washed peat, sand and sabudani were tried and their effect on shoots obtained was studied. In all cases MS medium was used.

The observations recorded are presented in the Table 14. The shoots did not grow well and subsequently dried on acid washed sand and peat in all cultivars.

Table 12: ANOVA for shoot multiplication in different genotypes of sugarcane with different levels of sucrose

Source of variation	DF	SS	MSS	F
Genotypes	2	5.40	2.70	7.21**
Sucrose levels	3	247.49	82.49	220.39**
Genotypes X Sucrose levels	6	20.57	3.43	9.16**
Error	108	40.42	0.34	

** Significant at P = 0.01

Table 13: Comparison of the genotypes and sucrose levels on shoot multiplication

Genotype	Mean (No. of shoots)	Sucrose levels (gl^{-1})	Mean (No. of shoots)
Co 8014	3.08	15	3.00
Co 85002	3.48	30	5.40
CoC 671	2.99	45	2.93
		60	1.38
SEm	0.10		0.11
CD (at 5 per cent)	0.27		0.30

Table 14: Effect of different supporting agents on *in vitro* multiplication in sugarcane

Sl. No.	Supporting agent	Average No. of shoots		
		Co 8014	Co 85002	CoC671
1	Peat	0	0	0
2	Acid washed fine sand	0	0	0
3	Sabudani (Sago)	1.5	1.0	0
4	Agar (control)	6.00	5.12	4.81

With sabudani as the gelling agent, the rate of multiplication was very low (1 - 1.5) compared to over five shoots in 25 days with agar gelled media in different cultivars. The shoots were very weak and could not grow at all, especially in 'CoC 671' where all shoots had dried within 25 days.

4.3 ROOTING OF SHOOTS

In vitro shoots (3 - 4 week old) devoid of roots were cultured on different media namely 1 gl $^{-1}$ activated charcoal (T1), 1 gl^{-1} activated charcoal + 60 gl^{-1} sucrose (T2), plain MS (T3), 0.25 mgl^{-1} NAA (T4), 0.5 mgl^{-1} NAA (T5), 1.0mgl^{-1} NAA (T6) and 2.0 mgl^{-1} NAA (T6) to induce roots. The frequency of rooting in different cultivars of sugarcane is presented in Table 15.

Frequency of rooting, 25 days after inoculation, varied from 10 to 100 per cent for the cultivar 'Co 8014'. Highest frequency of rooting (100 per cent) was seen in the treatments: *viz.*, 0.5 mgl^{-1} (T5), 1.0 mgl^{-1} (T6) and 2.0 mgl^{-1} (T7) NAA (Plate 10). T1 and T2 (1gl^{-1} activated charcoal and 1 gl^{-1} activated charcoal + 60 gl^{-1} sucrose) recorded the lowest frequency of rooting (10 per cent). The result also shows that rooting frequency was also high (90 per cent) in the treatment with plain MS (T3) and 0.25 mgl^{-1} NAA (T4).

The response pattern in 'Co 85002' was similar to 'Co 8014' (Plate 11). Rooting was 100 per cent with 1 mgl^{-1} and 2 mgl^{-1} NAA, respectively, followed by 90 per cent in 0.5 mgl^{-1} NAA, 70 per cent with 0.25 mgl^{-1} NAA. There was no rooting at all either in 1 gl^{-1} activated charcoal alone or with 60 gl^{-1} sucrose.

Table 15: Rooting frequency of shoots on different media in different genotypes of sugarcane

Genotypes	Treatment	Rooting (> 3 healthy roots)	Per cent rooting
Co 8014	T_1	1	10
	T_2	1	10
	T_3	9	90
	T_4	9	90
	T_5	10	100
	T_6	10	100
	T_7	10	100
Co 85002	T_1	0	0
	T_2	0	0
	T_3	7	70
	T_4	9	90
	T_5	9	90
	T_6	10	100
	T_7	10	100
CoC 671	T_1	0	0
	T_2	0	0
	T_3	6	60
	T_4	7	70
	T_5	7	70
	T_6	7	70
	T_7	6	60

No. of shoots used = 10

T_1 = Activated charcoal ($1 gl^{-1}$)

T_2 = Activated charcoal ($1 gl^{-1}$) + sucrose ($60 gl^{-1}$)

T_3 = Plain MS

T_4 = NAA ($0.25 mgl^{-1}$)

T_5 = NAA ($0.5 mgl^{-1}$)

T_6 = NAA ($1.00 mgl^{-1}$)

T_7 = NAA ($2.00 mgl^{-1}$)

Rooting response of 'CoC 671' was lower than that of 'Co 8014' and 'Co 85002' (Plate 12). The highest response of 70 per cent rooting was in 0.25 mgl^{-1}, 0.5 mgl^{-1} and 1.0 mgl^{-1}NAA. There was no response at all in 1 gl^{-1} activated charcoal alone or with 60 gl^{-1} sucrose. The nature of rooting was similar in all the cultivars it was similar with treatments within the cultivar also (Plate 13).

4.4 ESTABLISHMENT OF PLANTLETS

The details on the survival of the rooted shoots in polybags, placed in glasshouse, with respect to all three cultivars have been furnished in Table 16. The frequency of survival of plants in glasshouse established well (plate 14) with high frequency. Establishment of plants was the highest (94 per cent) in cultivars 'Co 8014'. Survival frequency was 88.0 and 72.0 per cent for 'Co 85002' and 'CoC 671' respectively.

Plate 10: Rooting of shoots in ‘Co 8014’ with different NAA levels

a. 0.5 mgl^{-1}
b. 1.0 mgl^{-1}
c. 2.0 mgl^{-1}

Plate 11: Rooting of shoots in ‘Co 85002’ with different NAA levels

a. 0.5 mgl^{-1}
b. 1.0 mgl^{-1}
c. 2.0 mgl^{-1}

Plate12: Rooting of shoots in ‘CoC 671’ with different NAA levels

a. 0.5 mgl^{-1}
b. 1.0 mgl^{-1}
c. 2.0 mgl^{-1}

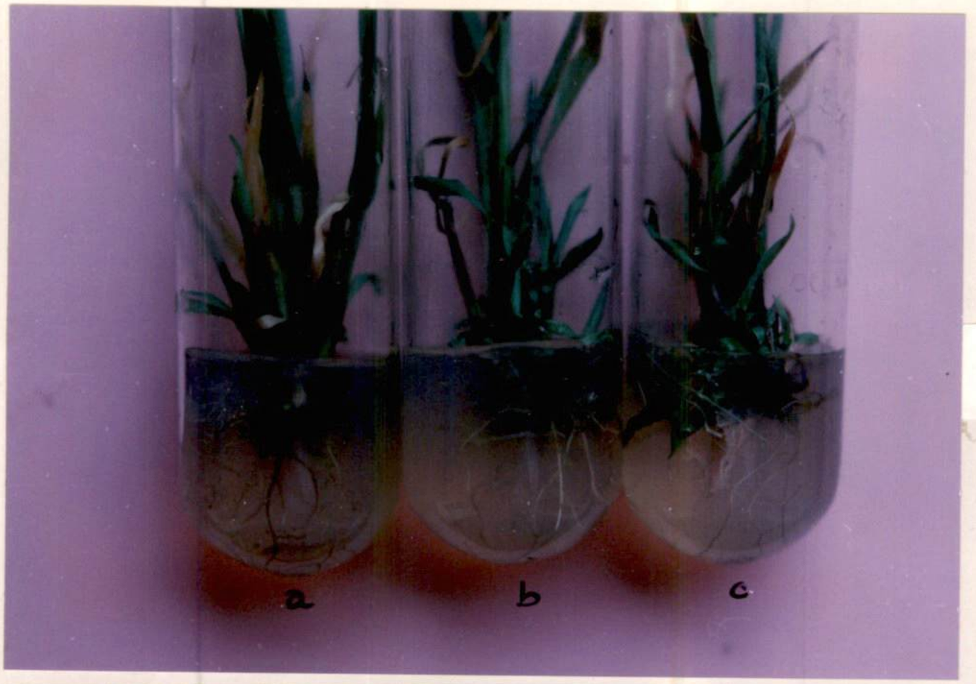

Plate 10

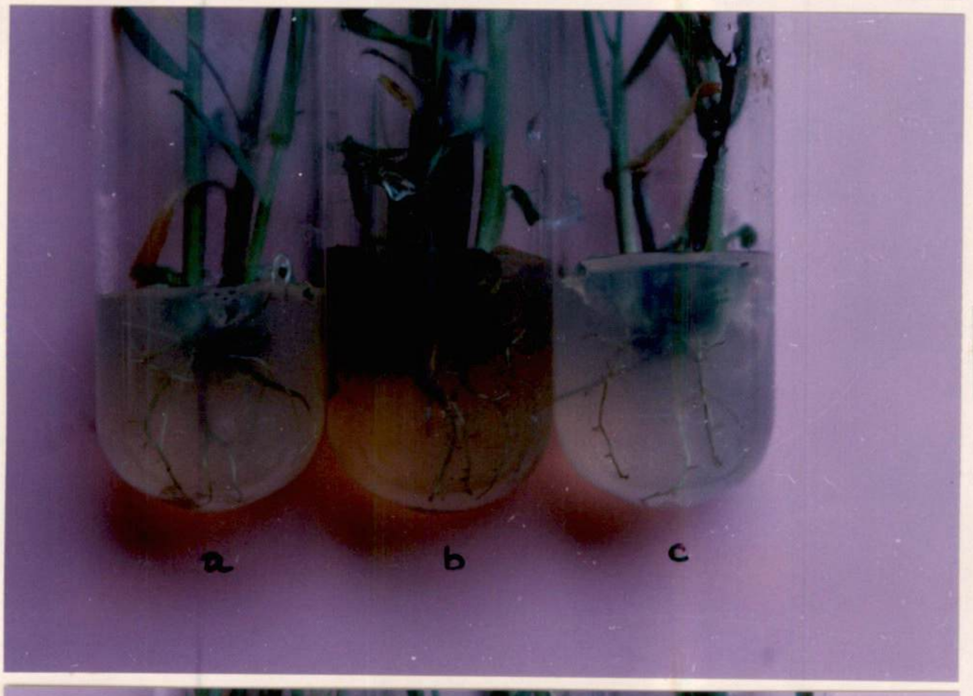

Plate 11

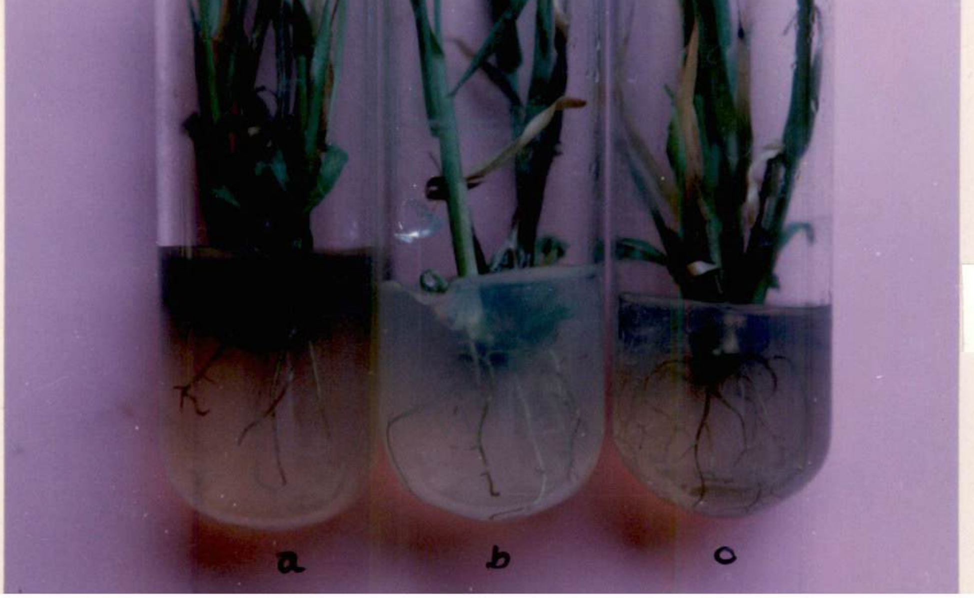

Plate 12

Plate 13: Photograph showing nature of rooting in 'Co 85002' with different levels of NAA; *viz.*,0.00, 0.25,0.50, 1.0 and 2.0 mgl^{-1} (from left to right)

Plate 13

Table 16: Survival of potted mericlones plants in glasshouse.

Cultivar	No.of Plants potted	No. of plants survived	Survival (%)
Co 8014	50	47	94.0
Co 85002	25	22	88.0
CoC 671	25	18	72.0

Plate 14: Established plantlets of 'Co 8014' in polybags (after 25 days)

Plate 14

DISCUSSION

V. DISUSSION

The commercial exploitation of plant tissue, organ and cell culture emerged several years ago with the development of 'shoot - tip' ('meristem') culture and recognition of the fact that the excised distal region of the apical shoot or the meristem was free from systemic infection from virus. The shoots obtained from such explants could then be induced to grow and proliferate in culture to produce large number of individuals. Obviously such plants retained the characteristics of the genotype from which the explants were taken. The procedure is now known as 'micropropagation'. This approach has since been applied to a large number of species, many of which are important crop plants in agriculture, forestry and horticulture. *In vitro* propagation now forms the basis of a growing industry, supplying high quality planting material, in vegetatively propagated species and also provide for an opportunity for genotype fixation in sexually propagated plant species of value.

Conventional methods of vegetative propagation have allowed unique heterozygous plants of many species to be multiplied and perpetuated for further breeding or for direct use as desirable cultivars. Micropropagation has several advantages over conventional vegetative propagation. It is a method of plant improvement by itself in many open pollinated, highly heterozygous and strictly sexually reproducing perennial tree species. Here the best available method as yet, is to identify elite individual genotype in natural stands and fix their genotypes through micropropagation.

It is highly probable that *in vitro* propagation techniques will soon become the preferred and commercially viable method for cloning desirable genotypes of forest and fruit trees, ornamentals and select F_1 hybrids. If production systems are cost - effective, *in vitro* micropropagation can offer space and time saving adavantages (Wang and Hu, 1982) besides providing clean planting material free from pathogenic bacteria, fungi, viruses, viroids, mycoplasma or rickettsia.

Sugarcane is a highly heterozygous complex polyploid with meiotic irregularities. It is vegetatively propagated through stem setts in large scale and the propagules are bulky, involving transportation across fields and regions. The risk of spreading pathogen is very high. This type of planting material may carry systemic pathogens and others that come along with the setts. Coupled with elimination of viruses, through meristem culture, micropropagation through axillary bud proliferation is desirable. This process eliminates systemic pathogens as well as ensures genetic uniformity of the planting material so generated.

Though micropropagated plants are desirable, they are not used for planting commercial surgarcane crop due to large number of plants needed per unit area and the costs associated with it. Yet,such plants can be used as a part of the seed sett multiplication cycle, preferably at the first stage itself. Though these plants can get infected subsequently, it would help in reducing the risks especially when planting material is moved from one place to another. In this study an attempt was made to reduce the cost of micropropagation in three popular cultivars; ‘Co8014’, ‘Co 85002’ and ‘CoC 671’.

Results obtained from various experiments conducted are discussed in this chapter.

5.1 INITIATION AND ESTABLISHMENT OF MERISTEM CULTURE

It was not until the early 1970s that the potential of shoot meristems as reproductive material was realised. The report of the successful meristem culture and multiplication of herbaceous strawberry (Boxus, 1974) and *Gerbera jamesonii* (Murashige *et al.*, 1974) were trend setters. In these cases shoot multiplication was achieved by added cytokinin.

In most plants, each leaf has an axillary meristem which has the capacity to develop into a shoot identical to the main shoot, under favourable conditions. Axillary meristems are inhibited to varying extent, according to the type of plant and how much it naturally branches, by apical dominance. Although the mechanisms of inhibition are complex and involve a number of hormones, the development of axillary shoots seems to be dependent ultimately on the supply and favourable balance of cytokinin (Phillips, 1975). Inclusion of a cytokinin in nutrient media promotes the growth of axillaries in cultured buds. In most cases benzyl adenine (BA) has been used for this purpose.

Like in several other crop species, in sugarcane also, BA has been the growth regulator of choice to initiate multiple shoots and further proliferation *in vitro* (Larkin, 1982). In the present study, maximum shoot induction and establishment of meristem cultures was with 2 mgl^{-1} in all three cultivars studied. The cultivar 'Co 8014' recorded the highest frequency of establishment (83 per cent) with 2.0 mgl^{-1} BA and 80 per cent establishment with 1.0 mgl^{-1}. The maximum frequency of establishment observed in 'Co 85002' and 'CoC 671' were 80 per cent and 72 per cent, respectively at 2.0 mgl^{-1} BA. Similar results were obtained by Dhumale *et al.* (1994) with 3 mgl^{-1} BA and 1 mgl^{-1} IAA. The study of Sreenivasan and Jalaja (1980) indicated that normal shoot development was obtained from two cultivars 'Co62171' and 'Co 7201' with 0.05 mgl^{-1} BA only. It reveals that concentration of BA required for shoot induction varies with genotypes. The amount of cytokinin applied and found adequate depends on the genotype used and the micropropagation strategy employed. Most investigators prefer to secure proliferation of shoots along with normal development of shoot from the cultured bud or the meristem. In such cases higher cytokinin levels have been used, wereas normal development of the shoot from the bud meristem might require very low levels of the growth regulators, as in the case of Sreenivasan and Jalaja (1980).

Shoot tips of varying in size from 0.1 to 2 mm in length were cultured with a view to raise virus - free plants.The size of the explant is critical for virus elimination since in most of the systemic viral diseases the pathogen often establishes a concentration gradient in different plant tissues. It has been generally observed that virus - free plants regenerated *in vitro* are inversely proportional to the size of the meristem cultured. The larger the tip, higher is the concentration of viral particles and the cells of apical dome are usually virus-free. Therefore, culture of shoot tips larger than 1mm may not ensure virus - free plants. Sreenivasan and Jalaja (1980) recorded virus free plantlets of two cultures 'Co62174' and 'Co7201' of sugarcane by using meristems of 0.5 mm length. In our investigation meristems of 1 mm to 2 mm in length have been used satisfactorily. Smaller explants are difficult to isolate with unaided eyes and are prone to damage. Visual observation of the plantlets and established plants indicated that they were free of viruses. However, this study was not specifically designed to demonstrate virus elimination.

One of the technical problems encountered in micropropagation is contamination of cultures. During large scale micropropagation of some plants certain types of slow growing microbial (bacterial) contaminants persist even after initial surface sterilisation of explants. Such contaminants (*Pseudomonas* sp., *Erwinia* sp. and *Bacillus* sp.) may persist for many generations without being noticed and cause reduction in vigour or chlorosis in micropropagated plantlets (Knauss and Miller, 1978). Addition of antibiotics or fungicides to the culture medium may control the infection by microbial contaminants. In our study also, there was problem of bacterial contamination during early establishment and during the first few subcultures. Contaminant - free cultures were maintained by carefully observing the culture tubes and discarding the suspected ones.

Browning of the medium and the explants is another problem encountered in micropropagation, frequently associated with micropropagation of woody perennials. It is due to the release of inhibiting phenolic substances in the growth medium during initiation of cultures. The problem of tissue browning and release of polyphenols is rare in sugarcane. But explants of 'CoC 671' were found to release dark brown pigment in the medium during shoot induction and establishment. The cultures with heavy release of dark - brown pigment seemed inhibited and the lower frequency of establishment of meristems in ' CoC 671' might be due to these pigments. Similar observations were made by Liu (1981, 1984). This problem could be reduced to a large extent by quick transfer of freshly cut explants to culture medium and / or by frequent transfer to fresh medium. In the other two cultivars this problem was not prominent and the frequency of establishment was also higher.

5.2 SHOOT MULTIPLICATION

Generally, three routes to micropropagation are recognised; enhanced release of axillary buds, production of adventitious shoots and somatic embryogenesis. In case of adventitious shoot formation, multiplication rate is very high. But the occurrence of somaclonal variation among the plantlets probably due to production of incipient callus is a draw back. Somatic embryogenesis also has similar disadvantages. In order to get true - to - type plants, the only option presently available is to secure enhanced release of axillary buds through meristem / vegetative bud culture.

Although the trate of multiplication is low in bud culture compared to other two cases, variation is nil or very less. Micropropagation coupled with apical meristem culture technique ensures the production of whole plants that are generally identical except those genotypes that are periclinal chimeras to start with. Even then one could easily sort out chimeras and multiply the desired type subsequently.

Sugarcane is a highly heterozygous, sexually sterile autopolyploid. It is propagated by setts. About 40 - 50,000 three budded setts are required for planting one hectare land. This method is followed in all the sugarcane growing regions of the world for planting commercial cane. The risks are too many. Besides clonal deterioration over a period of time, vegetative sett might harbour pathogenic fungi, bacteria, viruses and mycoplasama. Soil borne pathogenic microbes are also carried through the setts. Besides, a considerable part of the cane area has to be set aside for seed purpose. Though micropropagation is an attractive proposition for sugarcane, the cost of micropropagated plant is presently too high to be practicable. However, micropropagated plants could be used as a part of the seed production cycle to start with and the setts derived are used for commercial plantations, after one or two cycle of propagation in field. Alternatively, cost of production of micropropagated plants can be reduced by better multiplication rate, substitution for costly components, automation of labour intensive steps and sponsorship by sugar industry, to some extent. Considering the requirement of 40 - 50,000 three budded setts (5-8 t) for planting a hectare of land and the present price of cane, availability of plants @ Rs. 0.23-0.34 (spacing of mericlones 1m X 0.45 m, cost of seed cane Rs. 1000 / t) is comparable to the cost of seed setts. Further, the amount of additional cane available for processing and the associated benefits to the society can be taken into account to provide certain amount of leverage to the micropropagation industry. A rough estimate based on; average seed multiplication rate of 15x, average seed rate of 5 t/ha, cane replacement cycle of three years and yield of seed cane as 65 t/ha indicate that nearly 2.3 per cent more cane will be available to the sugar industry from the same project area if micropropagated material is used to plant commercial cane. However, the immediate task should be to promote the use of micropropagated plants to raise stage-I seeds from which stage-II and stage-III seeds (for commercial planting) can be sourced.

One of the means of reducing the cost of micropropagated plants is by increasing the rate of shoot multiplication, thereby reducing the cost per plant produced. In general, BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by kinetin and 2 - ip. In the present study, three levels of BA (0.5, 1.0 and 2 mgl^{-1}) and one treatment with 2 mgl^{-1} BA + 1 mgl^{-1} NAA combination were used to study their effect on shoot multiplication.

The treatment, 2 mgl^{-1} BA, recorded the highest (20.09) overall rate of multiplication followed by 1.0 mgl^{-1} BA (6.26).In all three genotypes, the treatment with 2 mgl^{-1} BA + 1 mgl^{-1} NAA was the least (3.0) effective. This is in contrast to the report of Alam *et al.* (1995) where 0.5-1mgl^{-1} BA and 4 mgl^{-1} NAA resulted in the best shoot proliferation. This might be due to different genotypes used in these studies.

All the three cultivars, *viz.*, 'Co 8014', 'Co 85002' and 'CoC 671' recorded the highest average rate of multiplication (20.05, 20.00 and 19.83, respectively) at 2 mgl^{-1} BA. Similar results were observed by Hendre *et al.* (1983a). But shoots obtained were weak, tiny and non-separable making it difficult to manage. Since rate of shoot multiplication is too high at this level of BA, there is a chance of getting *de novo* shoots through incipient callus, which goes unnoticed. The resultant plants may show somaclonal variation which is not desirable in any plant species.

Callus production can be induced in explants derived from meristem culture. From this callus, adventitious shoots,protocorms or embryoids can be induced to differentiate in large quantities in numerous species. Adventitious organogenesis may enable a substantially faster cloning than axillary shoot multiplication. But chromosome aberrations and ploidy changes occurred frequently when an intermediary callus phase was induced in the mass cloning process (Glendening and Sjolund, 1988; Kane *et al.*, 1988).

It is evident from the foregoing discussion that in order to ensure genetic stability of the plants propagated by microprpagation, intermediate callus formation or *de novo* shoot formation should be avoided. Precautions are obviously necessary to avoid unknowingly recycling mutant shoots and using levels of growth regulators that might induce adventitious shoots and callus. In a number of species, differences in the uniformity of plants propagated by axillary shoots and through callus are evident from reports in literature. *Asparagus* multiplied by Murashige *et al.*(1972) through stimulation of axillary buds were true - to - type, while those regenerated from callus by Malnassy and Ellison (1970) induced up to 70 per cent polyploids. Uniform potato plants are now commercially produced from shoot cultures but those regenerated adventitiously show a wide range of somaclonal variation (shepard *et al.*, 1980).

Therefore, it was considered undesirable to use 2 mgl $^{-1}$ BA for sugarcane, although multiplication rate was the highest (20.09). Here plants were tiny and in closely associated packs, indicating that at least some of them might be *de novo* shoots formed out of incipient callus or proliferation of the growing point. At this rate of multiplication genetic changes are quite possible, beating the very objective of micropropagation i.e. production of true - to -type plants. Normally the multiplication rate of 5 - 10 per month is sufficient to produce large number of shoots, economically in most species.

Among the other treatments, the one with 1 mgl^{-1} BA recorded better overall rate of multiplication (6.26). Another with 0.5 mgl^{-1} scored 4.43 overall rate of multiplication. In both these cases the plantlets were of desirable quality; well grown, easily separable and healthy.

For further confirmation of the sustainable rate of multiplication, these two treatments (1.0 mgl^{-1} and 0.5 mgl^{-1} BA) were used during II cycle of shoot multiplication. The mean rate of multiplication was more with 1 mgl^{-1} BA in all three cultivars i.e. 6.27, 4.91 and 4.61 in 'Co 8014','Co 85002' and 'CoC 671', respectively than at 0.5 mgl^{-1}BA. There was a slight reduction in the rate of multiplication in all the cultivars in the second cycle at comparable levels of BA. The difference in the shoot multiplication may be attributed to genotypic effects . Yet, the overall rate of multiplication was safe and desirable (5.27) at this level of BA. The plantlets obtained were strong, healthy and easily separable. This rate of multiplication is less risky and easy to manage. Here the shoots were from axillary buds and the chance of production of adventitious shoots or incipient callus is very low. This treatment (1 mgl^{-1} BA) was considered as safe and economical. At this rate of multiplication one can produce enormous number (2×10^{8}) of plants from a single bud in a year over a 4 - 5 week micropropagation cycle.

Another treatment (0.5 mgl^{-1}BA) resulted in low overall rate of shoot multiplication (3.27). The cultivars 'Co 8014', 'Co 85002' and 'CoC 671' scored 4.66, 2.86 and 2.58 average rate of multiplication, respectively. Here the plantlets were also of inferior quality compared to that obtained with 1 mgl^{-1} BA. Considering the quality of the plantlets and economy, this treatment is not suggested for large scale shoot multiplication.

For every litre of medium prepared, sucrose @ 30 g / l costs about Rs. 22.50 for analytical grade sugar. It appears unnecessary to use this costly source, though it is the est. Many commercial micropropagators use diamond sugar (Rs. 60 -

80 / kg). To further reduce the cost, commercial sugar (Rs. 15 / kg.) could also be tried. By using commercial sugar @ 30 g / l, one can reduce the cost of sucrose for every litre medium prepared from Rs. 22.50 to Rs 0.45.

In our experiments there was no statistical differences among the source of sucrose, *viz.*, analytical grade, laboratory reagent grade, diamond sugar or commercial sugar, with respect to average rate of multiplication. However, commercial grade sugar resulted in numerically lower rate of multiplication which might be due to undefined impurities present, especially the heavy metals.

The mean rate of shoot multiplication in control (AR) was 5.51 followed by LR grade (5.47), diamond sugar (5.32) and ordinary sugar (5.21) across all three cultivars. All four treatments resulted in strong, healthy and easily separable shoots, which could be subcultured easily. Considering the economy of micropropagation, it is suggested to use cheaper sources of sucrose like diamond sugar if not ordinary (tea) sugar in place of AR or LR grade sugar. The studies of this kind are hardly reported in literature, though important. As such the present thesis appears to be the first report showing the usefulness of commercial sugar for micropropagation at least in sugarcane. This kind of studies are required for other commercially micropropagated plant species also. Initial research on standardisation of protocols could be done with analytical grade sucrose. At the final stages of defining the protocol for commercial purpose, other sources of sucrose could be tried for possible utility.

Sucrose is the only energy available to *in vitro* shoots, as they can derive energy through photosynthesis to a very limited extent. Therefore, adequate supply of sucrose in the medium is essential. While lower levels of sucrose might limit growth, higher levels can create osmotic stress and lead to suppression of tissue differentiation and growth. Optimum level of sucrose is likely to differ with species but it is usually in the range of 20 to 60 gl^{-1}. In the present study the mean rate of multiplication across the cultivars was 5.40 at 30 gl^{-1} sucrose. But other treatments (15 gl^{-1}, 45 gl^{-1} and 60 gl^{-1}) resulted in very low overall multiplication rate (3.0, 2.93 and 1.38, respectively). Desirable plantlets were obtained with 30 gl^{-1}, whereas other treatments resulted in plantlets of poor quality. Considering the mean rate of shoot multiplication and quality of plantlets obtained, 30 gl^{-1} sucrose is the best for shoot multiplication. Similar observations were made by Shukla *et al.* (1994) who stated that 3 per cent sucrose was optimum for shoot establishment and proliferation higher levels of sucrose appear to have resulted in osmotic stress, while 15 gl^{-1} may be too inadequate.

Autotrophic microprpagation is an area which needs to be explored to reduce the cost of production, as no sugar is required in the medium. Kozai *et al.* (1987) have demonstrated that small plantlets are able to change from heterotrophic to mixotrophic, and then to autotrophic provided the CO_2 concentration is increased. Such studies are needed in sugarcane to reduce the cost of the medium to a large extent.

Agar is used as gelling / supporting agent in routine tissue culture studies. It is one of the costliest part of medium, which costs Rs. 1700 /kg. Thus it costs around Rs. 15.00 for every litre of medium prepared (@ 9 g / 1). In this investigation an attempt was made to replace agar by alternative supporting agents, *viz.*, acid washed fine sand, peat and sabudani. Response observed with peat and sand was nil overall three cultivars and the shoots dried within a week. With sabudani as the gelling agent, the rate of multiplication was very low (1-1.5) compared to over five shoots with control (agar) in different cultivars.

This study demonstrated the non feasibility of acid washed fine sand, peat and sabudani as supporting agents for shoot multiplication of sugarcane cultivars. This might be due to lack of aeration or compactness of the medium. In sabudani, a thick layer was formed immediately after cooling, which might inhibit shoot proliferation. Further investigations on alternative supporting agents are needed to replace agar, to make micropropagation cost - effective.

5.3 ROOTING OF SHOOTS

Most micropropagation protocols depends on a separate step for rooting of shoots, encouraging shoot proliferation alone during multiplication phase with the help of higher levels of cytokinins. Rooting of shoots during multiplication phase is both inconvenient and wasteful. At the level of BA used in the present study, shoots did not possess roots. Shoots devoid of roots were cultured in different media with 1 gl^{-1} activated charcoal + 60 gl^{-1} sucrose and 0 - 2 mgl^{-1} NAA to induce roots. Rooting can be achieved either by transferring the shoots to medium lacking cytokinin with or without a rooting hormone. All cytokinins inhibit rooting, and BAP does so particularly strongly. Rooting is also improved in many woody and herbaceous species by lowering the concentration of macro salts to a half or less. This is analogous to the report of Mohatkar *et al.*(1993), who obtained profuse and healthy rooting of sugarcane shoots on half strength of MS liquid medium.

Auxin is essential for root initiation in many species. However, high auxin concentration has been reported to inhibit the root elongation phase (Thimann, 1977). Contrary to this general observation, many workers reported that 5 mgl^{-1} NAA was good for rooting in sugarcane (Larkin, 1982; Shukla *et al.*, 1994; Alam *et al.*, 1995; Islam *et al.*, 1996). In this study 0.5 to 2 mgl^{-1} NAA concentrations resulted in profuse rooting in all cultivars. The cultivar 'Co 8014' recorded 100 per cent frequency of rooting with 0.5, 1.0 and 2.0 mgl^{-1} NAA. The treatments T_6 (1 mgl^{-1} NAA) and T_7 (2 mgl^{-1} NAA) gave 100 per cent rooting in 'Co 85002'. But maximum frequency of rooting observed in 'CoC 671' was 70 per cent with 0.25, 0.5 and 1.0 mgl^{-1} NAA. Interestingly, the treatment T_7 (2.0 mgl^{-1} NAA) resulted in low frequency of rooting (60 per cent) compared to other treatment with low NAA levels. This might be due to inhibition of rooting at higher concentration of NAA as discussed earlier.

The MS medium without growth regulators also resulted in sufficient rooting (60 - 90 per cent). Dobariya (1994) reported profuse rooting in the cultivar 'CoC 671' with 1 gl^{-1} activated charcoal + 60 gl^{-1} sucrose. On the contrary, this treatment (T_2) along with 1 gl^{-1} activated charcoal alone (T_1) was found unsuitable in our study. The observed differences might be attributed to the culture history of the shoots employed for root induction. In the studies of Dobariya (1994), callus derived shoots regenerated on a medium lacking growth regulators were used. The callus itself was induced and maintained on MS medium with 2,4 - D. In the present study auxins were not at all applied during the multiplication phase.

The present investigation revealed that normal MS medium supplemented with low levels of NAA (0.25 - 2.0 mgl^{-1}) induce strong and healthy rooting. The cultivar 'Co8014' responded well to all most all treatments compared to other two cultivars.

Debergh and Maene (1981) pointed out that rooting *in vitro* may represent the most labour intensive part of micropropagation, because of the need to manipulate shoots on an individual basis rather than in the clusters used during the proliferation stage. Roots formed *in vitro* do not adapt easily to normal conditions without some delay in growth. The most efficient methos of rooting is to directly transfer shoots into sterile blocks, tubes or pots (made of peat, inert mineral or plastic) after dipping in rooting hormone. The direct transfer of *in vitro* produced shoots to nonsterile sand (Rajbhandary and Bajaj, 1991) has been successful in a number of plant species. In this method, the intermediate step of first transferring the shoots to an auxin - containing medium is skipped, resulting in low cost. Such technology has to be studied in sugarcane also.

5.4 ESTABLISHMENT OF PLANTLETS

Hardening of plantlets is an important step in micropropagation. Sreenivasan and Sreenivasan (1980) observed 90-95 per cent survival of the plantlets in glasshouse, under shade. They used potting mixtures of sieved sand, silt and ratoon pressed mud in 1:1:1 proportion. The plantlets with good root system were taken and transplanted into potting mixture by trimming the leaves. Potting mixture used by Dobariya (1994) resulted in more than 90 per cent establishment. It had biogas slurry, soil and sand in 2:1:1 proportion. The same mixture was used in this study also.

Temperature and humidity are the two key factors, which control survival rate of plantlets in glasshouse. The largest problem concerns the drop in relative humidity from near to 100 per cent in the culture vessels to much lower values in the glasshouse. Absence of epicultcular wax on *in vitro* shoots leads to excessive water loss. Earlier, in this study when potted plants were raised in open glasshouse, under summer conditions, maximum (70 per cent) failure of the plants was recorded. This might be due to very high temperature (35-38°C) and low humidity (55-65 per cent) during that period. Temperature around 25-28°C and relative humidity around 75-90 per cent (under rainy conditions) resulted in maximum survival. The highest survival frequency (94 per cent) was observed in the cultivar 'Co 8014'. Other two cultivars, 'Co 85002' and 'CoC 671' recorded the survival frequency of 88 and 72 per cent respectively. Dobariya (1994) observed 92.4 per cent survival in 'CoC 671'. Variation in per cent survival is attributed to environmental factors. However, high frequency establishment (> 90 per cent) is desirable as it is one of the key factors in determining the final cost of production.

FUTURE LINE OF WORK

Micropropagation of sugarcane is achievable in any cultivar of sugarcane, and its utility has also been realised in raising a healthy and productive plantation. The main factor between adoption of this technology in the vegetative seed cycle of sugarcane cultivars is the cost. In order to reduce the cost per plantlet, some of the following areas of research can be considered.

1. The present study has shown that commercial grade sucrose can be used without adverse effect on the rate of multiplication and quality of plantlets. The cheapest of sources of unprocessed sucrose happens to be the sugarcane juice itself. One

could try fresh sugarcane juice diluted to desired level of sucrose and adjusted for the various nutrient elements and use it in place of sucrose (AR) in MS medium.

2. Alternative gelling agents and physical supports like filter paper bridges can be used to completely avoid agar which is one of the costliest part of the medium along with sucrose (AR).

3. Presently, in India, all tissue culture operations are done manually and technical labour is the most expensive part of the process. One way of reducing the cost of labour is to achieve higher rate of multiplication so that number of transfers (subcultures) are reduced for given number of plants finally desired.The present rate of multiplication (about 6 plantlets / 25 days) is high enough. Yet, if higher rate multiplication is achieved without the induction of callus and too much of over-crowding is desirable. However, with higher rate of multiplication it is necessary to test the plants for their true-to-type nature as there is a possibility of somaclonal variation in such cases.

4. *In vitro* rooting is a tedious procedure involving transfer of shoots to large number of culture vessels and other requirements like media, labour and other resources. It is worth while to try and induce rooting *ex vitro*, combining it with hardening and establishment phase. Alternatively, we could induce minimum amount of rooting during the penultimate cycle of multiplication phase by properly adjusting cytokinin and auxin levels and transfer such plantlets to net pots.

5. Autotrophic micropropagation is an area which needs to be explored to reduce the cost of media by applying the principles of hydrophonics.

SUMMARY

VI. SUMMARY

Investigations were carried out in three popular cultivars of sugarcane (*Saccharum* spp. hybrid), *viz.*, 'Co 8014', 'Co 85002' and 'CoC 671' with the purpose of developing a generalised protocol and to economise micropropagation. Apical meristems (dome) were used as explants in this investigation. Murashige and Skoog (1962) nutrient medium with 30 gl^{-1} sucrose was employed to support growth of cultures.

Three levels of BA (0.5, 1.0 and 2.0 mgl^{-1}) were used for establishing meristem cultures. Establishment was the best with 2.0 mgl^{-1} BA in all the cultivars; 83 per cent, 80 per cent and 72 per cent in 'Co 8014', 'Co 85002' and 'CoC 671', respectively.

Among the various combinations and concentrations of BA and NAA tried for shoot multiplication one with 1.0 mgl^{-1} BA was found to have reasonably high average rate of shoot multiplication (6.26) across all the cultivars. The shoots were strong and healthy. The same was confirmed during II cycle of shoot multiplication. Higher concentration of BA (2.0 mgl^{-1}) resulted in the highest shoot multiplication rate (20.09) overall the cultivars.But the plantlets were of inferior quality. The combination of BA and NAA recorded very low multiplication rate as well as weak and tiny plantlets.

Different sources of sucrose were tried as an alternative to analytical grade (AR) sucrose. All the four sources of sucrose, *viz.*, laboratory grade sucrose,

diamond sugar and ordinary sugar including control (AR) were found to be equally effective for shoot multiplication in all the three cultivars studied. One can use the cheapest sources of sucrose like ordinary sugar to make the micropropagtion cost - effective without affecting the rate of shoot multiplication.

Among the different levels of sucrose tried, one with 30 gl^{-1} resulted in highest overall shoot multiplication rate (5.40).The plantlets obtained were of desirable quality. The treatment with 15 gl^{-1} sucrose recorded low multiplication rate as well as plantlets of poor quality in all three cultivars. Higher levels of sucrose (45 and 60 gl^{-1}) gave very few but thick shoots.

Alternative supporting agents namely peat,sand and sabudani were tried in place of agar. But all these supporting agents failed to achieve the desired rate of shoot multiplication. In most cases plantlets dried within a weak.

Different media were tried to induce rooting of shoots.Very high rooting frequency was observed with 0.5, 1.0 and 2.0 mgl^{-1} NAA. Frequency of rooting was 100 per cent with 0.5, 1.0 and 2.0 mgl^{-1} NAA in 'Co 8014'. In 'Co 85002',100 per cent rooting occurred with 1.0 and 2.0 mgl^{-1} NAA and 0.5 mgl^{-1} NAA recorded 90 per cent rooting frequency. The best frequency of rooting recorded with 'CoC 671' (70 per cent) was with 0.25, 0.5 and 1.0 mgl^{-1} NAA.These treatments produced healthy, moderately thick and 3 - 4 cm long roots in 3 - 4 weeks. Good response was observed with plain MS and 0.25 mgl^{-1} NAA also. Interestingly, the two treatments with 1 gl^{-1} activated charcoal failed to induce rooting in most of the cases .

The potting mixture used in this study contained biogas slurry, soil and sand in 2:1:1 proportion. The survival of the potted plants with optimum temperature and RH in glasshouse was maximum (94 per cent) for the cultivar 'Co 8014' followed by 'Co 85002' (88 per cent) and 'C0C 671' (72 per cent).

Further investigations are needed on the aspects like, use of sugarcane juice as source of sucrose, alternative gelling agents, *ex vitro* rooting etc. to achieve micropropagation.

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MICROPROPAGATION IN SUGARCANE
(*Saccharum* spp. hybrid)

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ABSTACT

Investigations on the micropropagation in sugarcane (*Saccharum* spp. hybrid) were conducted at the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, College of Agriculture, Dharwad during 1996-98 in three popular cultivars of sugarcane, *viz.,* 'Co 8014', 'Co 85002' and 'CoC 671' with the purpose of developing a generalised protocol and to economise micropropagation. Apical meristems (domes) were used as explants.

Among three levels of BA used, one with 2 mgl^{-1} resulted in maximum establishment of the meristem culture in all three cultivars. Out of the various combinations and concentrations of BA and NAA tried, the treatment 1.0 mgl^{-1} BA was found to result in reasonably high average rate of shoot multiplication (6.26) across all the cultivars and the shoots were also of desirable quality. The treatment with 2.0 mgl^{-1} BA gave the highest average rate of shoot multiplication (20.09). But the plantlets were very tiny, weak and non-separable. All four sources of sucrose, *viz.,* analytical grade, laboratory grade, diamond sugar and ordinary (commercial) sugar were found to be equally effective for shoot multiplication in all three cultivars studied. The sucrose at 30 gl^{-1} resulted in the highest over all shoot multiplication rate (5.40), which is significantly superior to other concentrations. Alternative supportive agents, *viz.*, peat, sand and sabudani were unsuitable as supportive agents as a replacement for agar in producing desired rate of shoot multiplication.

Very high rooting frequency was observed with 0.5, 1.0 and 2.0 mgl^{-1} NAA in all three cultivars. Good response was also observed with plain MS and 0.25 mgl^{-1} NAA. The survival of the potted plants during rainy season in an uncontrolled glasshouse with RH of 88 per cent was maximum (94%) for the cultivar 'Co 8014' followed by 'Co 85002' (88%) and 'CoC 671' (72%).