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**MOLECULAR BASIS OF DIFFERENTIATION IN
PLANT TISSUE CULTURE
WITH
SPECIAL REFERENCE TO SUGARCANE**



A THESIS SUBMITTED TO THE UNIVERSITY OF POONA FOR
THE DEGREE OF
DOCTOR OF PHILOSOPHY
(IN CHEMISTRY)

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*This thesis is dedicated to my
Grandfather
Because of his untiring encouragement,
I could complete my education
He ought to have been alive to - day
Memories of him are like a sentinel of
Light in the dark*

C O N T E N T S

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ACKNOWLEDGEMENTS

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DECLARATION

Certified that the work incorporated in the thesis "Molecular Basis of Differentiation in Plant Tissue Culture with Special Reference to Sugarcane" submitted by Shri Ratikant Raghunath Hendre was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

P.K. Ranjekar

(P.K. Ranjekar)
Research Guide

LIST OF ABBREVIATIONS

min	:	minutes
h	:	hour
kg/sq.cm.	:	kilogram per square centimeter
mg/l	:	milligram per litre
nm	:	nanometer
RPM	:	Revolution Per Minute
SDS	:	Sodium dodecyl sulphate
A	:	Adenine
G	:	Guanine
T	:	Thymine
C	:	Cytosine
5-mC	:	5-methyl cytosine
SSC	:	Saline Sodium Citrate (0.15 M NaCl, 0.015 M Tri Sodium Citrate, pH 7.0)
v/v	:	volume per volume
O.D.	:	Optical Density
mA	:	milliampere
EDTA	:	Ethylene diamine tetra acetic acid
kbp	:	kilobase pairs
np	:	nucleotide pairs
Ms	:	Moles second per litre
pg	:	Pico gram (10^{-12} g)
HAP	:	Hydroxyapatite

HRTD : High resolution thermal denaturation

HPLC : High performance liquid chromatography

Tris : Tris-hydroxymethyl amino methane

PIPES : Piperazine-N-N'-bis 2 ethane
sulfonic acid

TE : Tris-EDTA buffer (10 mM Tris-HCl,
pH 7.5, 1 mM, EDTA)

TAE : Tris-Acetate-EDTA buffer (10 mM Tris-
20 mM acetic acid, 2 mM EDTA, pH 8.1)

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ABSTRACT

Genesis of the problem

It is now generally accepted that eukaryotic DNA is dynamic and undergoes selective rearrangements and changes during cell differentiation. In plants, variations in complexity and composition of nuclear DNA have been reported during cell differentiation (Nagl and Rucker, 1976, Buiatti, 1977; Verma and Dougall, 1978, Durante et al. 1983; Ssymank, 1984; Duhrssen et al. 1984). Differential replication of satellite DNA and main band DNA has also been described during dedifferentiation in carrot (Hase et al. 1979) and melon (Grisvard et al. 1980). The rDNA, however, has been shown to undergo no gross change during dedifferentiation in carrot and Jerusalem artichoke explants (Hase et al. 1982).

In our laboratory, the tissue culture work of sugarcane was undertaken as early as in 1971 with an objective of producing virus free sugarcane through stem tip culture. During this work, callus tissues were obtained from two sugarcane cultivars, namely CO-740 and CO-7219. Callus obtained from the former cultivar retained the capacity for differentiation even after 10 years of its subculturing whereas the latter lost its capacity for differentiation within 1 year of subculturing. Moreover, differentiation could be easily

induced in CO-740 callus cultures by omitting auxin from the medium and incubating them in the presence of light (12 h/day photoperiod). Finally, multiple sugarcane shoots without roots could be propagated vegetatively by in vitro techniques, thus making it easier to get both callus and shoot tissues for further work.

Using the above sugarcane system, profound biochemical changes were shown to occur in callus cultures during differentiation (Khan et al., 1984; Dhamankar and Mascarenhas, 1984; Dwivedi et al., 1984; 1985; Rawal et al., 1985). However, no information was available with respect to changes in DNA during differentiation in this system. The DNA studies were, therefore, undertaken and these included characterization of DNA with respect to its T_m , buoyant density, content and distribution of 5mC, content of repetitive DNA and arrangement of repetitive and single copy DNA sequences.

Objectives

The specific objectives in the present work were as follows:

1. To standardize conditions for rapid multiplication of sugarcane plants.

2. To analyze restriction enzyme digestion patterns and to study the methylation status of sugarcane DNA from the above three systems.
3. To assess the nature, copy number and kinetic complexity of repetitive DNA sequences and their size distribution in CO-740 callus and shoot, and CO-7219 callus.
4. To study physicochemical characteristics of total and repetitive DNA in CO-740 callus and shoot, and CO-7219 callus.
5. To analyse the patterns of genome organization i.e. arrangement of repetitive and single copy DNA sequences in two callus systems and CO-740 shoot system.

Organization of thesis

The entire thesis has been organized in six chapters.

Information given in the Chapter "General information about sugarcane" is essentially based on published literature on sugarcane: its history, taxonomy, physiology, morphology, propagation and economic importance.

In Chapter I, the method of establishing callus cultures of two sugarcane cultivars namely CO-740 and

CO-7219 and results of rapid multiplication of sugarcane using stem tip are described.

Chapter II summarizes results of studies on isolation of sugarcane DNA from the two callus systems and shoot system, and determination of base composition of total DNA by thermal denaturation and isopycnic centrifugation. Information about base composition heterogeneity of sugarcane DNA was obtained by high resolution thermal denaturation. The quantitation of 5-methyl cytosine is also detailed in this chapter.

Results of studies on reassociation kinetics of sugarcane DNAs from the three tissues are detailed in Chapter III.

Chapter IV deals with the physico-chemical characterization of sugarcane repetitive DNA using thermal denaturation, high performance liquid chromatography and reassociation kinetics of isolated enriched repetitive DNA components isolated from differentiated and dedifferentiated tissues.

Studies on arrangement of repetitive and single copy DNA sequences in sugarcane genome from the two callus tissues and shoot tissues are described in Chapter V.

Chapter VI gives an overview of all the results obtained during the course of the present studies on in vitro differentiation in sugarcane.

References are given at the end of each chapter. The list of publications/papers presented is given at the end of the thesis and the reprints of two publications are enclosed.

Summary of thesis

Development of a method for rapid multiplication of sugarcane using stem tip culture

A method of in vitro rapid multiplication of sugarcane has been described. In this method, shoot tips of sugarcane cultivar CO-740 were first cultured on a basal medium containing growth regulators. The elongated shoot tips were then transferred to a second medium supplemented with kinetin and benzyl amino purine. Rooting was next induced by transferring individual shoots to a rooting medium. Within 2 weeks, 80% of the plants developed a root system. By means of transfers at regular intervals, it was possible to produce about 200,000 sugarcane plants in 6 months from a single shoot tip. These plants were successfully grown in the field. This method could permit cane breeders to multiply new sugarcane cultivars in a short period of time.

Assessment of changes in native sugarcane DNA in differentiated and dedifferentiated tissues

Physico-chemical characterization of native sugarcane DNAs was carried out to assess some gross molecular

changes in differentiated (CO-740 shoots) and de-differentiated tissues (CO-740 callus and CO-7219 callus). A few important results from these studies are as follows:

- (i) The melting temperature of the three sugarcane DNAs was in the range of 86.2°C to 86.5°C. The first derivative melting profiles were polyphasic and indicated skewing on G + C rich side
- (ii) The DNAs from the two callus tissues (CO-740 and CO-7219) banded as a single narrow peak with a buoyant density of 1.702 g/cm³ while CO-740 shoot DNA banded at 1.703 g/cm³.
- (iii) CO-740 shoot DNA was more digested with BamHI, EcoRI, AluI and HindIII as compared with the digestions of two callus DNAs. The three sugarcane DNAs did not show any organized repeat structure with these enzymes. HpaII digested all the three sugarcane DNAs to a lesser extent than the digestion with MspI indicating less methylation of 5'-CCGG-3' sequences.
- (iv) The 5 mC proportion was found to be 4 moles percent in CO-740 callus and shoot DNAs while CO-7219 callus DNA contained about 9 moles percent of 5 mC. Thus, although similarities were observed in melting temperature and buoyant density, there were a few changes with respect to digestion with restriction enzymes and content of 5 mC in total sugarcane DNAs in callus and shoot tissues.

Study of kinetic complexity of repeated and single copy DNA sequences in total sugarcane DNA from callus and shoot tissues

Non-linear regression analysis of reassociation curves of native, sonicated sugarcane DNAs from two callus cultures, namely CO-740 and CO-7219 and shoots of CO-740 revealed the presence of one fast and one slow DNA component, each reassociating with second order kinetics. A comparison of rate constant of these two DNA component indicated that the fast reassociating DNA fraction consisted of repeated DNA sequences while slow reassociating DNA fraction included mainly single copy DNA sequences. Marked differences were noticed in the amount, kinetic complexity and size distribution of repeated DNA sequences in callus and shoot tissues. For example, in CO-740 callus, the fast DNA comprised 47.5% of the total DNA as against 16.5% in CO-740 shoot and 38.5% in CO-7219 callus. The proportion of the slow component was 25-30% in all the three DNAs. Its kinetic complexity was in the range of $2.5-4.4 \times 10^8$ nucleotide pairs in both the callus cultures and was significantly higher than that in shoot DNA (6.1×10^6 nucleotide pairs). The reassociation kinetics data thus revealed that repeated DNA sequences undergo a significant change during the in vitro differentiation of callus tissue to shoot

in sugarcane.

Physico-chemical properties of repetitive DNA from callus and shoot tissues of sugarcane

- (i) To assess the kinetic heterogeneity of repetitive DNA fractions from callus and shoot tissues of sugarcane, miniCot curves of isolated Cot 100 DNA fractions were determined. The data indicated the presence of very fast and fast reassociating DNA components in CO-740 callus and shoot tissues and only fast reassociating DNA component in CO-7219 callus.
- (ii) The thermal denaturation analysis of repetitive DNA showed the presence of one low and one high melting DNA component: in all the three types of sugarcane tissues. Further, it was observed that the high melting component was confined to Cot 0.1 fraction only.
- (iii) High resolution thermal denaturation showed that the number of thermal components varied from 34 to 47 in the repeat DNA of the three sugarcane tissues and that there was an extensive rearrangement in repetitive DNA during the in vitro differentiation of callus tissue to shoot in sugarcane.
- (iv) HPLC analysis of S1 nuclease resistant repetitive DNA revealed that the content of 5 mC was 2.7, 2.7 and 1.9 moles percent in CO-740 callus, CO-740 shoot and CO-7219 callus respectively. An additional peak was

seen in CO-740 callus and CO-740 shoot DNAs, its amount being 1.1 moles percent and 4.2 moles percent respectively. This additional peak was not seen in CO-7219 callus DNA. A comparative study of sugarcane repetitive DNAs from callus and shoot tissues thus revealed a genomic rearrangement as well as differences in the content of 5 mC and presence of odd bases.

DNA sequence organization in callus and shoot tissues of sugarcane

DNA sequence organization patterns were studied in the three sugarcane tissues namely CO-740 callus, CO-740 shoot and CO-7219 callus. The reassociation kinetics of 10 kbp long DNA fragments showed that 75-86% of the sugarcane genomes consisted of alternating single copy and repetitive DNA sequences. The length of interspersed single copy DNA sequences varied in a narrow range of 1.4-2.2 kbp. The size distribution of S1 nuclease resistant repetitive duplexes isolated from long unsheared DNA fragments revealed the occurrence of two major size classes of repeats (>1.5 kbp and 0.7 kbp to 1.1 kbp). The third size class (<0.2 kbp) was also present though in small amounts (6-20%). Extensive variation was observed in the proportions of the different size classes of interspersed repetitive DNA sequences in the three different tissues of sugarcane.

The DNA sequence organization in sugarcane genome (1C = 4 pg) is thus mixed type and this substantiates the trend in plants, namely, plants with 1C nuclear DNA content of less than 5 picograms have diverse patterns of sequence organization while those with 1C nuclear DNA content greater than 5 picograms have predominantly a short period interspersed pattern.

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GENERAL INFORMATION ABOUT SUGARCANE

HISTORY

Sugarcane is an important agricultural commodity for export and domestic consumption in many countries in the world. It has been known from the earliest times and the historical records go back into the remote days of ancient civilizations which flourished long before the Christian era. The use of gur, a product obtained from sugarcane, in religious practices indicates the presence of sugarcane plant in India since the days of mythology. The earliest mention of sugarcane is found in Indian Writtings 1400-1000 B.C. Barber mentions that no such references are found in ancient Chinese or Egyptian records (Purseglove, 1972).

The derivation of all names for sugar and sugarcane comes from the Sanskrit word called "Shakkar" which means 'a new crop from the East'. The word Ikshvaku occurring in Ramayana refers to a celebrated family of Kings, ancestors of Rama, and is derived from the word Ikshu meaning sugarcane.

Foreign travellers to India, two thousand years ago, have mentioned sugarcane in their travel notes. Sugar and sugarcane were seen by Alexander the Great during the conquest of India in 326 B.C. He carried sugarcane to Europe on his retreat from India in 325 B.C. There had also been a chance introduction of sugarcane to Persia, Arabia

and Egypt. Arabs took canes from India and travelled to Greece via Asia minor around 8th century. In the first century A.D., Dioscorides described sugarcane as a honey called Sakkaron collected from reeds in India and Arabia Felix, with consistency of salt and which could be crunched between teeth. Sugarcane was introduced to China in about the fourth century A.D.

TAXONOMY

According to Hutchinson (1973), sugarcane is classified in the genus Saccharum, tribe Panicoideae, subtribe Andropogoneae and family Gramineae (Fig.I.1). The Saccharum genus has only five species: (1) S. spontaneum (2) S. officinarum (3) S. robustum (4) S. barberi and (5) S. sinense. A brief description of each of these five species is as follows:
S. spontaneum L. ($2n = 40-128$).

These are wild canes and are widely distributed. They vary in size from thin to thick types and occur in rocky to water logged locations. They also occur under extremes of climate. The darbha grass is a wild form of sugarcane and played a very significant role in the evolution of new commercial varieties by hybridization and selection techniques. All the commercially cultivated varieties of sugarcane today in India may be said to have S. spontaneum as an ancestral parent; directly or indirectly, to a smaller or larger extent.

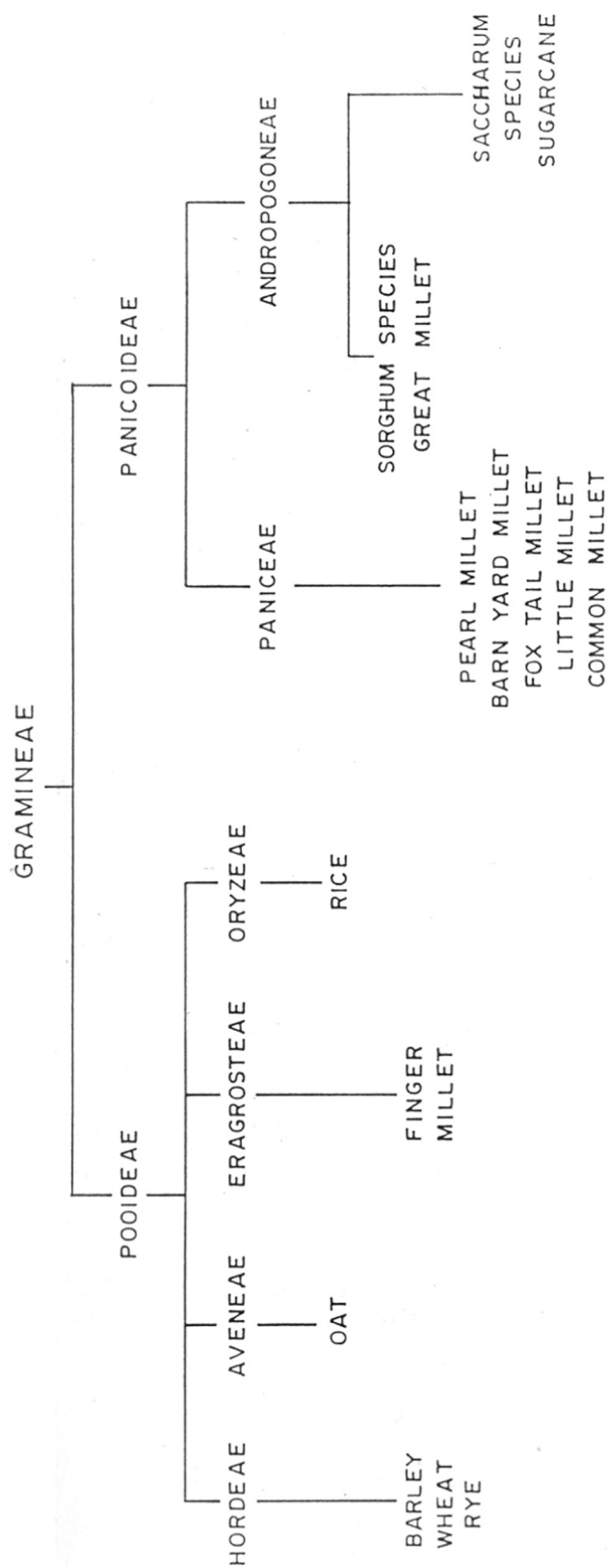


FIG. I·1. CLASSIFICATION OF
GRAMINEAE PLANT SPECIES ACCORDING TO HUTCHINSON

Hutchinson, J. (1973). In the families of flowering plants, 3rd ed., Oxford University Press, Oxford

S. saccharum L (2n = 80)

According to Brandes and Artschwager (1948), origin of Saccharum officinarum is in New Guinea. It was quite possible for a primitive Saccharum to reach New Guinea from Asia and undergo modifications that did not occur elsewhere. In the latter half of 18th century, it migrated from the Pacific Islands. Because of their thick stems, cultivars of this species are called noble canes to differentiate them from the thin canes of S. barberi and S. sinense. They cross with S. spontaneum and back crossing to the noble canes is known as nobilization. This is probably an indication that S. officinarum is of recent origin in an evolutionary scale and that man has selected and protected it for its sugar content by rearing it under his care. Modern canes are derived mostly from the germ plasm of 20 nobles and derivatives of S. spontaneum (Arcenaux, 1967). S. officinarum is only suited to tropical conditions and requires a favourable soil and climate for its performance. The stems are stout and thick. The canes have a high content of sucrose and low content of fibre. Culms are pale or dark green to dark yellow, red or purple, some times striped. Leaves are long and broad. Inflorescence rachis are without long hair or glabrate and with usually three glumes. The canes are susceptible to most of the major diseases including mosaic, sereh, streak, red rot, root rot and gummosis.

S. robustum - Brades and Jeswiet ex Grassl ($2n = 60 - 194$)

These canes occur in New Guinea and have been recently discovered and classified during the expedition led by Jeswiet. They grow along riverbanks in the wet tropics. It is surmised that this species might have played an important role in nature in the evolution of the species of S. officinarum.

S. barberi - Jeswiet ($2n = 82-124$)

The thin canes of North India are grouped here. They probably were in cultivation from immemorial times. They are found in Indochina, Taiwan and Southern China. S. barberi is suited to subtropical and temperate climates and is most widely cultivated in North India. The canes are short, medium to slender in thickness, high in fibre content, medium in sucrose content and rather poor in yields. They, however, produce tillers abundantly. The canes are greenish, white or ivory colour with cylindrical nodes. Their leaves are narrow and the inflorescence rachis have long hairs. S. barberi clones are resistant to major diseases such as sereh, streak and gummosis, but susceptible to root rot and mosaic disease. Hybridization with wild S. spontaneum may have occurred to produce the thinner and harder canes of S. barberi, thus providing forms with resistance to frost, diseases and pests.

TH-568

S. sinense - Roxb ($2n = 118$)

Some of the canes cultivated in North India and South Eastern Asia are grouped here. They were called 'Chinese cane' as they were collected by Roxburgh in 1796 in Canton, China. S. sinense can be grown in subtropical and temperate countries.

PHYSIOLOGY AND MORPHOLOGY

Climate

Sugarcane is a crop which requires high temperature good sunlight, plenty of water i.e. at least 1500 mm of rain per year unless grown with irrigation; and fertile soil unless amply supplied with fertilizers and good drainage. The ideal climate is the one with a long and warm summer growing season and a fairly dry, sunny and cool but frost free ripening and harvesting season. A relatively long growing period, ranging from 10-18 months is essential for high yields. Sugarcane flowers under the influence of long nights when the cane is reaching maturity.

Stem

Stem is known as cane or culm. It is solid, 2.5 - 6 meters in height, usually erect and unbranched except for tillers at base. It is composed of a series of joints 5 - 25 cms long and is 1.5 - 6 cms in diameter consisting of nodes and internodes.

Inflorescence

Inflorescence is known as arrow or tassel (Fig. I.2).

581.09

FIGURE I.2 SUGARCANE SHOWING INFLORESCENCE
AND OTHER PARTS

- A. Young plants growing from sett (x 1/10)
- B. Portion of stem (x 1/4)
- C. Setts showing early growth
- D. Base lamina (x 1/2)
- E. Inflorescence (x 1/10)
- F. Portion of inflorescence (x 5)
- G. Spikelet (x 10)
- H. Floral diagram

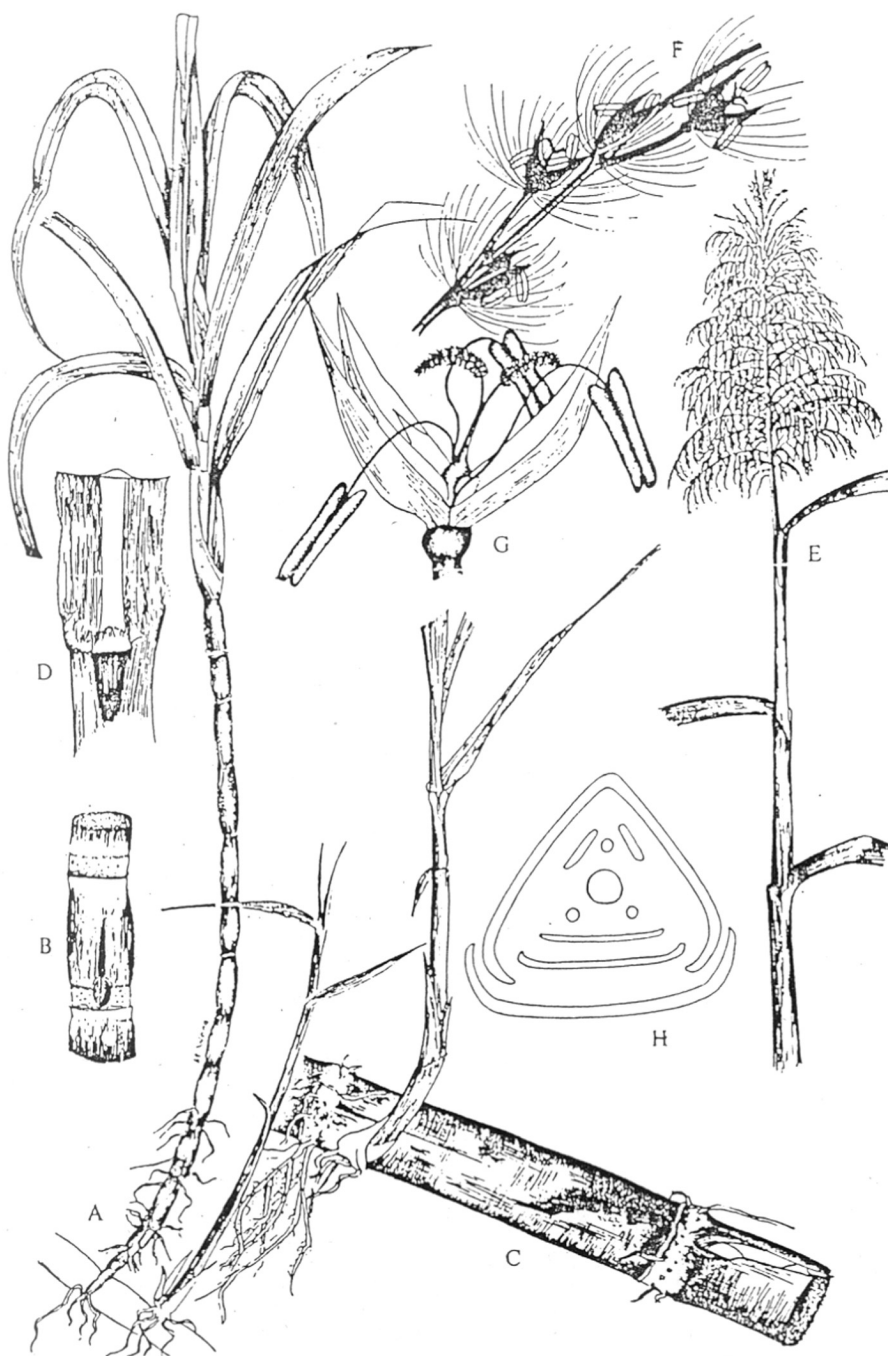


FIG. I. 2.

When a cane has reached maturity, it can change from a vegetative to a reproductive phase under specific conditions. The terminal meristem ceases to form leaves and is transformed into an inflorescence primordia about three months before the inflorescence appears.

Ploidy

The Saccharum genus is genetically a complex one. It is characterised by variable chromosome numbers and high polyploidy. It hybridizes easily with other genera and multiplies vegetatively. Bremer (1962) prefers S. robustum as subspecies of S. officinarum. These are octoploids with a basic number of ten. Sugarcane with higher chromosome number are near tropics and with less chromosome number are near sub-tropics and temperate zones.

PROPAGATION

As seed formation is rarely seen in sugarcane, it is propagated vegetatively by using stem cuttings, also known as seed setts, seed cane or seed pieces. The cuttings are usually taken from plant canes 8 - 12 months old. They should be free from diseases and pests and with two or more buds or eyes.

The choice of a suitable cultivar of cane depends upon characters such as yield of cane, clarity of juice, growth rate and disease resistance. Most breeders use a wide range of parent types to produce maximum variability. High yielding seedlings usually appear as chance segregants.

Dr. Barber, the economic botanist of Madras State, utilized the S. spontaneum of Coimbatore and the S. officinarum types 'Vellai' and raised a batch of hybrid seedlings in 1918. By 1923, the Sugarcane Breeding Institute at Coimbatore released under its nomenclature a new variety namely CO-205 which created a new epoch in the history of sugarcane and sugar industry in India. CO-740 is a high yielding variety and was released in 1949 (Parthasarathy, 1972).

ECONOMIC IMPORTANCE

Sugarcane is a major agricultural crop in tropical and sub-tropical regions of the world and an important export product in many developing countries. World sugar output in 1980-81 was 86.97 million metric tonnes. Of this, 61% was produced from sugarcane (Lichts 1981). Brazil, Cuba and India are the major producers of sugarcane. Table I.1 gives a list of cane sugar producing countries in the World and their annual production of sugarcane in 1980-81 season.

TABLE I.1: WORLD CANE SUGAR PRODUCTION DURING 1980-1981

Country	Production (x 1000 metric tonnes)
Brazil	8350
Cuba	6000
India	5600
Australia	3430
Mexico	2790
United States	2653
Philippines	2500
China	2250
South Africa	1800
Argentina	1700
Thailand	1550
Indonesia	1440
Colombia	1265
Dominican Republic	1050
Hawaii	950
Taiwan	757
Egypt	675
Pakistan	570
Mauritius	550
Peru	520
Guatemala	480
Fiji	425
Other Countries	5938
Total	53243

(Lichts, F.O., 1981).

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CHAPTER I

DEVELOPMENT OF A METHOD FOR RAPID MULTIPLICATION OF
SUGARCANE USING STEM TIP CULTURE

SUMMARY

A method of in vitro rapid propagation of sugarcane has been described in this Chapter. In this method, shoot tips of sugarcane cultivar CO-740 were first cultured on a basal medium supplemented with growth regulators where they elongated. These elongated shoot tips were then transferred to a second medium supplemented with kinetin and benzylamino purine on which 15-20 shoots were produced from a single shoot tip within 2-3 weeks. For root initiation, these shoots were separated and transferred to a rooting medium. Within 2 weeks, 80% of the plants developed a healthy root system. By means of transfer at regular intervals, it was possible to produce about 200,000 plants in 6 months from a single shoot tip. These plants were successfully grown in the field. This method could permit cane breeders to multiply new cultivars in a short period of time. In case of CO-740 callus, shoot differentiation capacity is retained even after 100 subcultures while CO-7219 callus tissue loses the differentiation capacity between 7-10 subcultures.

INTRODUCTION

The term 'Plant tissue culture' broadly refers to the in vitro cultivation of all plant parts (a single cell, a tissue or an organ) on a defined nutrient medium (Biondi and Thorpe, 1981). Thus any given plant tissue composed of living cells is a suitable explant for the initiation of a plant tissue culture. Recent advances in the field of plant, cell and tissue culture have resulted in the utilization of this technology for basic research in the chemical and biochemical sciences and also for practical applications to several agricultural and horticultural problems. The use of in vitro techniques for asexual propagation is one of the most advanced areas of plant tissue culture.

A major stimulus for application of plant tissue culture techniques to the propagation of several plant species may be attributed to (i) the early work by Morel (1960) on the propagation of orchids and (ii) the development and wide spread use of a new medium with high concentration of mineral salts by Murashige and Skoog (1962). Following the success with rapid propagation of orchids, significant number of other plants such as Gerbera (Murashige et al. 1974), Gladiolus (Hussey, 1975, Ziv et al., 1970), ginger (Hosoki and Sagawa, 1977; Nadgauda et al., 1980), strawberry (Boxus, 1974; Kartha et al., 1980), carnation (Earle and Laughous, 1975), Iris (Hussey, 1976), teak and eucalyptus (Gupta et al.

1980, 1981) have also been propagated using this technique. Hussey (1978) and DeFossard (1976) have listed several applications of rapid propagation techniques for (i) plants which arise as single plants during hybridization for testing and eventual commercial production, (ii) vegetative propagation of 'difficult to propagate' species (iii) year around propagation of clones, and (iv) propagation of genetically uniform parent plants in large number.

Rapid multiplication of plants can be achieved by induction of adventitious shoots, somatic embryogenesis; and development of axillary buds, terminal buds and shoot tips (Hughes, 1981). Among these, rapid multiplication using stem tips is very important as plants developed from shoot tips are very similar to the mother plants both phenotypically and genotypically (D'Ammato, 1977; Mellor and Stace-Smith, 1977; Morel (1964). In sugarcane, a serious problem in conventional sugarcane breeding is that it takes several years before any newly isolated variety can be commercially released.

Before I describe my work on clonal propagation of sugarcane, I would like to summarize in brief the tissue culture work done in sugarcane.

Tissue culture work in sugarcane has been critically reviewed by Heinz et al. (1977) and Liu (1981, 1984).

Research on sugarcane tissue and cell culture was first started in 1961 by Nickell in Hawaii, U.S.A. Since then research work on sugarcane tissue culture has been initiated in many countries.

The first reports of callus induction, suspension culture and differentiation in sugarcane appeared in 1969 (Heinz and Mee 1969, Nickell and Maretzki, 1969). Later, Maretzki et al. (1974) published results on nutritional and biochemical studies using cell suspension cultures. The initial site of callus induction in young leaf and sub apical meristem explants was first identified by Liu et al. (1982). The configuration of shoot differentiation pattern in callus was first discovered in 1974 (Liu and Chen, 1974). This was also the first report among all the Gramineous crops. With the advent of protoplast techniques, sugarcane protoplasts were obtained from callus (Maretzki and Nickell 1973) and from suspension culture (Chen and Shih, 1982). Recently Srinivasan and Vasil (1986) have reported plant regeneration from sugarcane protoplasts. Vasil et al. (1979) were first to study nitrogen fixation in test tube by establishing an association between sugarcane callus and nitrogen fixing bacterium Azospirillum.

Regeneration of plantlets from sugarcane anther culture has also been reported (Chen et al., 1979,b). It

should be mentioned that Krishnamurthy (1974) was first to obtain a disease resistant plant for crop improvement using cell culture methods while Liu and Chen (1978a,b) were the first to obtain several high-yielding and high-sucrose clones derived from callus cultures. Information summarised in Table 1.1 shows how tissue cultures of sugarcane have been used for developmental, biochemical, physiological, cytological, mutational and pathological studies.

When the present work was undertaken, there was no known method for the rapid multiplication of sugarcane in vitro. If such method is standardised for sugarcane, it could be of great utility to breeders for propagating new cultivars within a short time. This chapter describes a method for the rapid multiplication of sugarcane cultivar CO-740 and successful transfer of such plants to the field.

TABLE 1.1: SUCCESSES IN SUGARCANE CELL CULTURE RESEARCH

Success	Reference
Callus induction and shoot differentiation	Heinz and Mee, 1969, 1970; Barba and Nickell, 1969; Liu, 1971; Lat and Lantin, 1976; Sauvaire and Galzy, 1980.
Root regeneration	Liu <u>et al.</u> 1972; Nadar and Heinz, 1977; Liu, 1981.
Suspension culture	Nickell and Maretzki, 1969; Heinz and Mee, 1970; Chen, 1978; Chen and Liu, 1982; Maretzki <u>et al.</u> 1974.
Histological examination of callus origin	Liu <u>et al.</u> , 1982.
Histological studies on organogenesis	Liu and Chen, 1974; Nadar <u>et al.</u> 1978; Ho and Vasil, 1983.
Genetic variability in suspension cells	Heinz <u>et al.</u> , 1969; Heinz and Mee, 1970; Liu <u>et al.</u> 1977.
Regenerated plants morphological characters	Heinz and Mee, 1971; Liu and Chen, 1976; Lat and Lantin, 1976.
Chromosome number in callus culture	Heinz and Mee, 1971; Liu and Chen, 1976.
Enzyme patterns in callus culture	Heinz and Mee, 1971; Liu <u>et al.</u> 1977; Liu and Chen, 1978a; Khan <u>et al.</u> 1984; Dwivedi <u>et al.</u> 1984, 1985; Rawal <u>et al.</u> 1985; Dhamankar and Mascarenhas, 1984.
Nitrogen fixation in callus	Vasil <u>et al.</u> , 1979.
Induced mutations in callus cultures	Heinz and Mee, 1970; Liu <u>et al.</u> 1977

TABLE 1.1 (continued)

Success	References
Protoplast isolation	Chen and Liu, 1974; Krishnamurthi, 1976; Evans <u>et al.</u> 1980; Maretzki and Nickell, 1973; Chen and Shih, 1983; Guiderdoni 1985.
Protoplast isolation and regeneration of shoots	Srinivasan and Vasil, 1986.
Agriculturally useful somaclone/ disease resistance	
Fiji disease	Krishnamurthi 1974; Krishnamurthi and Taskal, 1974.
Downy mildew	Krishnamurthi, 1974; Chen <u>et al.</u> 1979a.
Eyespot disease	Heinz <u>et al.</u> 1977; Larkin and Scowcroft 1981.
Smut disease	Liu 1981.
Mosaic virus free	Mascarenhas <u>et al.</u> (1973); Hendre <u>et al.</u> (1975).
High yielding	Liu and Chen, 1978a, b; Liu 1981.
High sucrose	Liu and Chen 1978b.
Salt-tolerant cell line	Liu and Yeh, 1982; Fitch and Moore, 1981.
Anther culture	Moore and Maretzki, 1974; Chen <u>et al.</u> 1979; Liu <u>et al.</u> 1980; Fitch and Moore, 1981.
Transformation of sugarcane protoplasts	Chen <u>et al.</u> 1987.

MATERIALS AND METHODS

Glassware and its sterilization

Only Corning or Borosil brand glassware was used in all the experiments. The glassware was cleaned by using 10% sodium carbonate and 30% nitric acid. First it was boiled in sodium carbonate solution for 2 h. After washing with tap water, it was immersed in 30% nitric acid overnight. Next day, it was washed with tap water and rinsed with distilled water. It was then allowed to dry on a draining rack at room temperature.

Mainly 25 x 150 mm test tubes and 250 ml Erlenmeyer flasks were used for all the experiments. After drying, they were plugged with sterilized absorbent cotton. Petridishes (80 x 10 mm) were wrapped in brown paper. All glassware and dissection instruments required for inoculations were steam sterilized at 1 kg/sq.cm pressure for 1 h followed by drying in an oven at 100°C for 2 h.

Chemicals

All the chemicals used throughout the work were of Analytical Reagent (AR) or Guaranteed Reagent (GR) grade and were obtained from British Drug House (BDH) (India), E. Merck (India), Glaxo (India) or Sarabhai Chemicals (India).

Coconut Milk

Coconut milk is a natural and generally accepted growth regulator. In our work, coconut milk was pooled from

several tender coconuts and filtered through cotton. It was autoclaved at 1 kg/sq.cm pressure for 20 min and stored at -20°C. When required, it was thawed, mixed thoroughly and used.

Media preparation

Barba and Nickell (1969) reported that White's medium was superior for sugarcane callus growth but was unsuitable for differentiation. They also found that Murashige and Skoog's medium was the best for differentiation in sugarcane callus. In our present studies, White's medium (1963) and Murashige and Skoog's medium (1962) were used and the composition of these media are given in Tables 1.2, 1.3 and 1.4. It will be noticed from these tables that the composition of the macro salts is according to the original reports with minor modifications while the alterations made in the micronutrients and organic constituents are as follows:

In White's medium (1963), ferric citrate was added at 5 mg/l concentration instead of ferric sulphate. Copper and molybdenum were added according to Smith (1967). Solution of Na_2FeEDTA was prepared according to Murashige and Skoog (1962) containing 5.57 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 7.45 g Na_2EDTA in one litre. Five ml of this stock solution was used per litre of the medium. Edamin and indole acetic acid were omitted from Murashige and Skoog's medium (1962) and 2% sucrose was added instead of glucose.

TABLE 1.2: INORGANIC SALTS USED IN DIFFERENT MEDIA

Macro elements

Chemical	Murashige & Skoog (1962) mg/l	White (1963) mg/l
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	-	288
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440	-
KNO_3	1900	80
KH_2PO_4	170	-
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370	720
NH_4NO_3	1650	-
Na_2SO_4	-	200
KCl	-	65
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	-	16.5

TABLE 1.3: INORGANIC SALTS USED IN DIFFERENT MEDIA

Macro elements

Chemical	Murashige & Skoog (1962) mg/l	White (1963) mg/l
Na_2FeEDTA	37.3	-
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8	-
Ferric citrate	-	5
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6	2.6
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3	7
H_3BO_3	6.2	1.50
KI	0.83	0.75
CuCl_2	-	0.27*
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25	0.25*
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025	-
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025	-

* indicates a difference from the original media

TABLE 1.4: ORGANIC CONSTITUENTS IN DIFFERENT MEDIA

Chemical	Murashige and Skoog (1962) mg/l	White (1963) mg/l
Thiamine HCl	0.1	1*
Pyridoxine HCl	0.5	1*
Nicotinic acid	0.5	5*
Glycine	2	30*
Inositol	100	-

* indicates a difference from White's vitamin solution
(White, 1963)

The medium for shoot elongation (MS-I) contained mineral salts and vitamins according to Murashige and Skoog (1962), supplemented with coconut water (5%), gibberellic acid (GA_3) (0.1 mg/l) and indole butyric acid (IBA) (0.01 mg/l). The medium for shoot multiplication (MS-II) also contained the mineral salts and vitamins according to Murashige and Skoog (1962) but was supplemented with 0.1 mg/l kinetin, 0.2 mg/l benzyl amino purine and 10% coconut milk. For callus initiation and maintenance, medium (MS-III) containing salts and vitamins according to Murashige and Skoog (1962), coconut milk (10% v/v), inositol (100 mg/l) and 2,4-Dichlorophenoxy acetic acid (3 mg/l) was routinely used. For shoot initiation, MS-III medium without 2,4-dichlorophenoxy acetic acid (MS-IV) was used. Liquid medium (WB-I) containing salts and vitamins according to White (1963) and 2% sucrose was used for rooting of sugarcane shoots.

Adjustment of pH and sterilization of media

Generally, plant tissue cultures are grown at pH 5.5 to 6.0. In this work, pH of the medium was adjusted to 5.6-5.8 using 0.1 N NaOH or 0.1 N HCl and the medium was solidified if necessary by adding 0.8% agar. The medium was steamed for 30 min to melt the agar and then was distributed into tubes (25 x 150 mm) in 20 ml aliquots. Liquid medium was directly distributed in 20 ml aliquots in

tubes or 50 ml aliquots in 250 ml Erlenmeyer flasks. This medium was then steam sterilized at 1 kg/sq.cm. pressure for 20 min and steamed for 30 min on the following day. Solutions of heat labile compounds were added after filtration through Millipore membranes (0.22 μ m pore size) to previously sterilized medium. Aqueous solutions of GA₃ and IBA were sterilized by Millipore filtration (0.22 μ m pore size).

Sterilization and culture of shoot tips and young leaves of sugarcane

Sugarcane plants (Saccharum officinarum L cultivar CO-740 and CO-7219) were grown in laboratory farms. Six to eight month old canes were selected for shoot tip culture. Stem segments (15-20 mm) containing the meristem were excised and first rinsed with water containing a detergent and then treated with 30% saturated chlorine water for 20 min. At each step, the explants were washed thoroughly with sterile water. Dissection of the individual segments was then carried out in a sterile cabinet. The sterile cabinet was cleaned with 70% alcohol and the ultraviolet tube in it was switched on for half an hour before inoculations. Each shoot tip included the apical dome and 3-5 leaf primordia, and was approximately 0.5-1.5 mm long and 1 mm in diameter. All the shoot tips were then transferred to tubes containing 20 ml of liquid basal media.

Here, two main media were used, one for shoot elongation and the other for shoot multiplication. For rooting, elongated shoots were transferred to test tubes containing rooting medium (WB-I) and Whatman No. 1 filter paper platforms. The purpose of filter paper platform was to support shoots in upright position.

Transfer of plants

Young plantlets with a well developed root system were removed from the culture tubes. The roots were thoroughly washed to remove traces of nutrients and sucrose. They were then transferred to pots (6 x 6 cm) containing sterile soil:vermiculite (1:1) mixture. The pots were kept in a cabinet having a 12 h photoperiod and were watered daily. Within two weeks after the opening of a new leaf, plants were transferred to the field. The extent of survival of plants which were transferred to field was 80%.

Transformation of sugarcane

(i) Bacterial growth

Virulent strains of Agrobacterium tumefaciens No.2146, 2147, 2148, 2232 and 2822 were obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune-8, India. These cultures were routinely maintained on a nutrient broth slant (peptone 1%; beef extract 1%; sodium chloride 0.5%). Before inoculation, the strains were grown on a reciprocating shaker at 30°C in liquid nutrient broth for 18 h.

(ii) Plant material

Sugarcane plants of variety CO-740 were obtained by sowing three nodal setts in the field. When plants were about 2 months old, they reached an height of 50-60 cm. Incision was made on stem at a distance of 15 cm above the ground and 0.1 ml cell suspension of A. tumefaciens in an exponential growth phase was applied with a Pasteur pipette. After application of Agrobacterium culture, the incision area was sealed with a strip of parafilm to minimize the transpiration losses from this region.

In the control experiment, tomato seedlings (variety Pusa Red) were raised from seeds and were infected with A. tumefaciens as described earlier. Each time, 10 plants were inoculated with each strain.

(iii) Octopine assay by paper electrophoresis

The best method of detecting transformation is the presence of octopine in plant extract. For the detection of octopine, we used the method of Otten and Schilperoort (1978) with modification. As T-DNA genes from Ti plasmid encode enzymes for the production of opines which are not normally synthesized by the host organism, the occurrence of such opines in plant cells is the evidence for the existence and expression of T-DNA.

To a small amount of tissue (60-100 mg) from infected area 50-100 μ l of acidified methanol was added and tissue

was ground in Eppendorf tube with a small glass rod for 5 min. The extract was microfuged and supernatant was immediately used for octopine assay.

At the start, 10-20 μ l of supernatant was spotted onto a 45 x 12 cm sheet of Whatman 3 MM filter paper and dried along with a 5 μ l of aqueous standard solution containing nopaline, octopine and arginine (1 mg/ml, each). Extracts of tomato crown gall and healthy stem tissue were also spotted, at a distance of 1 cm as a positive and negative control, respectively. Prior to electrophoresis, the filter paper was wetted with electrophoretic running buffer (5% formic acid, 15% acetic acid and 80% water). Electrophoresis was then performed at 400 V for 1 h after which the filter paper was thoroughly air dried. The air dried filter paper was dipped in a staining solution (1:1 solution of 0.02% phenanthrenequinone in 100% ethanol and 10% NaOH in 60% ethanol), air dried and examined under long wave UV light (302 nm).

R E S U L T S

RAPID MULTIPLICATION OF SUGARCANE USING SHOOT TIPS

In view of its high yield and high sucrose content, sugarcane cultivar CO-740 is widely cultivated in the Western India. However, this cultivar is invariably infected with sugarcane mosaic virus. To obtain virus free plants by tissue culture method, apical meristem and shoot apex (apical meristems with 1-3 leaf primordia) explants are generally used. In the present work, stem segments (15-20 mm) containing shoot tip were sterilised and the latter were dissected as described in 'MATERIALS AND METHODS' and were inoculated into tubes containing 20 ml of liquid MS-I medium. Figure 1.1 shows the actual shoot tip on zero day. Contamination was a serious problem in this step. Though all precautions were taken to avoid contamination, over 50% of the shoot tip cultures were discarded because of contamination. To control the contamination, 5 mg each of tetracycline and aureofungin (From Hindustan Antibiotics Ltd., Pimpri, India) were added per litre of medium. MS-I medium was found to be optimum for the growth of virus free sugarcane shoot tips (Hendre et al., 1975).

Shoot tip elongation

For elongation of the cultured shoot tips, it was essential to provide appropriate photo-period and

FIGURE 1.1 SUGARCANE SHOOT TIP CULTURED
ON MS-I MEDIUM (ZERO DAY)

FIGURE 1.2 ELONGATED SUGARCANE SHOOT TIP
ON MS-I MEDIUM (2 WEEKS AFTER CULTURE)



FIG. 1-1.



FIG. 1-2

temperature. This was achieved by incubating the culture in a growth chamber provided with a 12 h/day photoperiod from cool, day light 40 watts fluorescent tubes situated about 10 to 15 cm. away from cultures (Illumination approximately 2000 lux). The temperature in the chamber was about 28°C under illumination and 25°C in the dark. Visual observations of the growth of shoot tips were made at regular intervals. Within a week's time, shoot tips were observed to become green. Approximately 75-80% of the cultures elongated and attained a height of about 25 mm in 15-20 days as seen in Fig. 1.2.

Multiplication of shoots

If elongated shoots were allowed to grow in MS-I medium, shoots will further elongate but will hardly form multiple shoots. For obtaining multiple shoots from a single shoot, it was, therefore, essential to change the growth condition and medium. Individual elongated shoot tip (20-25 mm) were transferred to 250 ml capacity conical flasks containing 50 ml of liquid MS-II medium. The flasks were kept on a rotary shaker (120 RPM) and were illuminated by fluorescent tubes (700 lux intensity) for 24 h at 30°C. In shake flasks, the shoot tips elongated further and attained a height of 40-50 mm in another 15 days. At this stage, the basal portion of leaves and the growth medium turned brown and there was a total inhibition of growth. Removal of the

brown leaves and frequent transfer of shoots to fresh medium on every two days for one week were found to be essential for continued viability of shoots. This step was essential only initially with the primary explants. By means of this treatment, the shoots survived and could later be subcultured regularly in shake flasks containing 50 ml of fresh MS-II medium.

FIGURE 1.3 shows the number of shoots formed after a few subcultures while Table 1.5 shows the rate of multiplication of shoots during subsequent subcultures. Shoots were separated individually and subcultured in fresh MS-II medium every 15 days. Table 1.5 shows that for the first two subcultures, the number of shoots obtained remains the same. This rate slowly increases reaching a ratio of 1:10 after the sixth subculture at 15 to 20 day intervals. The multiplication rate further increases to 1:20. It was of interest to note that the multiplication rate and vigor of growth of shoots did not diminish even after 50 subcultures.

Rooting of sugarcane shoots

Multiple shoots obtained by rapid multiplication in flasks were healthy but did not develop roots. This is because the concentration of phytohormones in shoot multiplication medium (MS-II) is such that only shoots will be formed but not roots. For root initiation and further development, it was thus necessary to change the medium.

FIGURE 1.3 MULTIPLE SUGARCANE SHOOTS (WITHOUT ROOTS)
GROWING IN MS-II MEDIUM



FIG. 1-3.

TABLE 1.5: RATE OF MULTIPLICATION OF SUGARCANE

Sub-culture	No. of shoots cultured	No. of shoots recovered	Multipli-cation rate	Time interval per passage(days)
1	30	30	1:1	15
2	18	18	1:1	15
3	16	65	1:4	15
4	8	33	1:4	15
5	12	110	1:9	15
6	15	148	1:10	15
7	22	266	1:12	15
8	18	280	1:15	15
9	14	249	1:18	15
10	23	465	1:20	15

Medium: MS-II

Inoculum: 1 shoot per flask

For this purpose, shoots were separated individually and inoculated into the tubes containing 20 ml of rooting medium (WB-I) and supported on a filter paper platform. Cultures were incubated in a growth chamber (12h/day photoperiod). Within fifteen days, 80% of the shoots developed a healthy root system with root hairs (Fig.1.4). Here, a few plants also developed tillers.

Transfer of sugarcane plants into the field

The next step in the present work was to transfer the rooted shoots to fields to obtain virus free plants. However, the plants obtained by in vitro methods are generally delicate and liable to die if transferred to field directly. Survival of plants in field can be achieved if test tube plants are acclimatised in a slow and step wise manner. For this purpose, rooted sugarcane plants were removed from test tubes, thoroughly washed with water to remove adhering medium and then transferred to pots containing 1:1 mixture of sterile soil and vermiculite. Individual plants were covered with beakers to maintain humidity and were kept for hardening at 25°C under the light of 800-1000 lux intensity for two weeks. Later on, plants were transferred to glass house for further acclimatization. When plants had grown to a height of 7.5-10 cm, they were planted in the field. More than 80% of the plants survived in the field (Fig.1.5).

FIGURE 1.4 SUGARCANE PLANT WITH ROOTS
(WELL DEVELOPED ROOTS FORMED ON
TRANSFER TO WB-I MEDIUM)

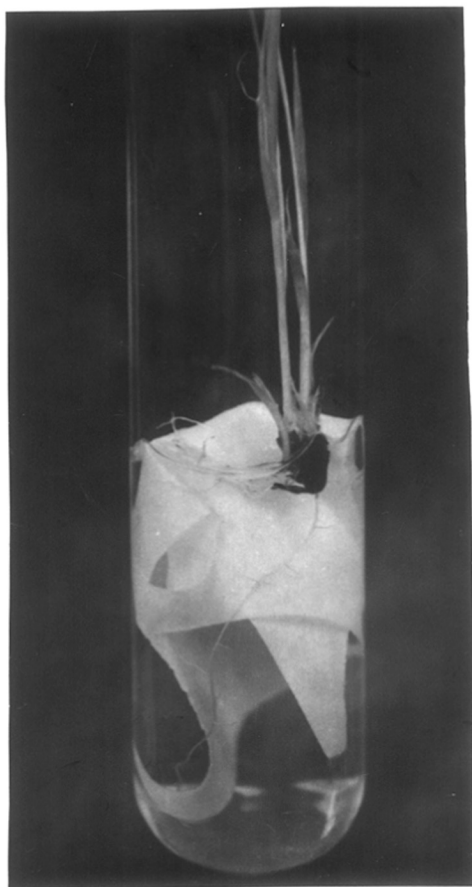


FIG. 1.4

FIGURE 1.5 SUGARCANE PLANTS, RAISED THROUGH
SHOOT TIP CULTURE, GROWN IN FIELD



FIG. 1.5.

All the experiments, starting from shoot tip to transplanting in the field were repeated four times with two cultivars namely CO-740 and CO-7219 and were found to be highly reproducible.

Field trials

Using the rapid multiplication technique, over 1000 sugarcane plants were planted in the field. To determine variations, if any, in these sugarcane plants, observations were made on 100 plants grown in a 11 x 11 meter plot and spaced 1 meter apart. Table 1.6 shows the average results of this experiment. From this Table, it can be seen that the plants show very little variation in the parameters such as height of the plants, number of internodes per cane, weight of individual cane and the number of canes per clump.

RAPID MULTIPLICATION OF SUGARCANE FROM CALLUS CULTURES

Regeneration of shoots from callus cultures

In the present work, I used two approaches for rapid multiplication of sugarcane plants. The first approach involves rapid multiplication of sugarcane plants using shoot tips while the second approach is shoot regeneration from callus cultures. A small clump of callus mass contains many thousand of cells. If proper conditions such as temperature, photoperiod and phytohormones are provided, theoretically, each cell should form a plant. In this way, large number of plants can be obtained in a short period of time.

TABLE 1.6: FIELD DATA ON CLONALLY PROPAGATED SUGARCANE PLANTS

Plot size (m)	11 x 11
Spacing (m)	1 x 1
Date of planting	June 16, 1980
Date of harvest	Sept. 30, 1981
Number of plants sown	100.0
Number of plants survived	83.0
Height of millable canes (m)	2.6 ± 0.024
Number of internodes/cane	30.0 ± 0.23
Number of canes/clump	18.0 ± 0.02
Weight of individual canes (kg)	1.1 ± 0.02
Number of canes per plot	1494.0

Standard error was determined by using the formula

$$\text{Standard error} = \pm \sqrt{\frac{\Delta_1^2 + \Delta_2^2 + \dots}{n(n-1) (n-2) \dots}}$$

where n is the total number of canes and Δ is the difference between the average value and the individual value.

In regeneration of shoots from callus culture, the first step is to obtain callus culture and the second step is the shoot formation.

Callus initiation

Formulation of an appropriate medium for aseptic purpose is the main step in any research work in plant tissue culture. Segments (0.5-1.0 cm) of the innermost young leaf were cultured on a medium MS-III solidified with 0.8% bacto agar. Before the present work was initiated, Nadar and Heinz (1977) had used the same medium for callus initiation in sugarcane.

For callus initiation, the sugarcane cultures were first incubated in total darkness at 25°C. After 5 to 8 days of incubation, callus cells were visible around the periphery of the leaf explant. Frequently, callus mass abruptly grew out from the lower epidermis. This method was repeated at least 6 times and was found to be applicable for CO-740 and CO-7219 sugarcane cultivars.

As nutrients and space were limited in test tube, callus mass grew only upto a certain limit and stopped growing further. This step which took about one month was called a stationary phase (Fig. 1.6). For healthy and perpetual growth, it was necessary to transfer a small portion of callus (150-200 mg) into tubes containing freshly prepared, sterilised medium MS-III and cultures were incubated at 25°C in total darkness. This step was repeated every month

FIGURE 1.6 SUGARCANE CALLUS SHOWING PROFUSE GROWTH
ON MS-III MEDIUM, 3 WEEKS AFTER SUBCULTURE

FIGURE 1.7 SUGARCANE CALLUS SHOWING INITIAL SIGNS OF
SHOOT FORMATION (GREEN SPOTS) WITHIN 1 WEEK
AFTER SUBCULTURE ON MS-IV MEDIUM



FIG. 1-6 .

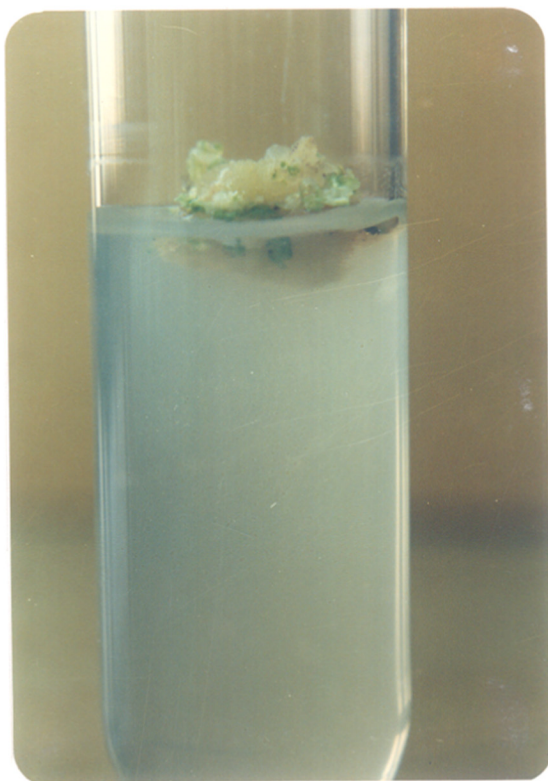


FIG. 1-7.

for the maintenance of callus cultures or till required for shoot initiation.

Shoot formation from callus cultures

By definition, callus is an unorganised mass of cells. When it is subjected to a sudden change in temperature, photoperiod and/or phytohormones, shoot initiation begins. In case of sugarcane callus, shoot initiation was accomplished by transferring callus to the medium MS-IV. Medium MS-IV is same as medium MS-III but without 2,4-dichlorophenoxy acetic acid. Callus cultures were incubated in the presence of light (12 h/day photoperiod, 1000 lux illumination). Within a week after transfer of callus to medium MS-IV, dark green spots are seen all over callus mass (Fig. 1.7). This is the early sign of shoot initiation. One week after this stage, leaf like structure, about 5 mm long, emerges from the green callus which can be clearly seen in Fig. 1.8. Figure 1.9 shows that shoots of about 1-1.5 cm long are formed in a month's time. Once shoots are formed, a dominance effect is observed on the remaining undifferentiated callus. Here, no further shoot differentiation is observed until non-differentiated tissue is separated from shoots and transferred to a fresh medium (MS-IV).

An interesting situation was encountered with respect to differentiation capacity of callus obtained from young leaves of sugarcane cultivars CO-740 and CO-7219. Inspite of

FIGURE 1.8 SUGARCANE CALLUS WITH MANY TINY SHOOTS
(TWO WEEKS AFTER INOCULATION ON MS-IV
MEDIUM)

FIGURE 1.9 SUGARCANE SHOOTS REGENERATED FROM CALLUS
(A MONTH AFTER SUBCULTURE ON MS-IV MEDIUM)



FIG. 1-8.



FIG. 1-9.

repeated subcultures for 5-6 years, CO-740 callus retained its capacity for shoot formation. There was no diminution in its vigor and the capacity for differentiation into shoots. But this was not the case with CO-7219 callus. For first five subcultures, there was no change in the capacity for shoot differentiation. But on further subculture, there was a gradual diminution in the vigour and the capacity for shoot formation and after about 10 subcultures differentiation capacity was completely lost (Data not presented). The callus which was earlier hard, compact and granular, became very soft, watery and friable on frequent subculture, and lost its capacity for shoot formation. In order to obtain shoots of CO-7219 cultivar regularly, it was, therefore, necessary to isolate fresh callus from time to time.

Rooting

Shoots regenerated from both the sugarcane callus tissues (CO-740 and CO-7219) were without roots. For root initiation, shoots were first separated from each other and adhering callus mass was discarded. The shoots were then transferred to liquid medium (WB-I) and roots were developed within 2 weeks time (Fig. 1.4). When root system was well developed, plants were transplanted in small pots and later into the field as described earlier. Under our experimental conditions, 75-80% of the shoots formed roots in 15 days after transfer to rooting medium and about 80% of the rooted plants survived on transfer to field.

TRANSFORMATION OF SUGARCANE BY AGROBACTERIUM TUMEFACIENS

In order to standardise conditions for transformation, tomato plants Var. Pusa Red were chosen as controls. Ten seedlings (20-25 cm height) were inoculated with each of the five different virulent strains of Agrobacterium tumefaciens namely 2146, 2147, 2148, 2232 and 2822 as described in 'MATERIALS AND METHODS'. Inoculation resulted in the swellings at the site of inoculation and two weeks later, small protuberances were seen. Figure 1.10 shows the formation of a typical crown gall tumor in tomato after about four weeks. Tumor formation was noticed with all the 5 strains of A. tumefaciens and more than 80% of the tomato seedlings formed tumors.

Sugarcane plants (Cv. CO-740), raised from seed setts, with a height of about 50-60 cm were infected with five strains of A. tumefaciens as already mentioned and were observed for tumor formation every week for two months. No visible tumor formation was observed.

It is known that after infection with A. tumefaciens, if there is no sign of tumor formation, it does not mean that plant transformation has not taken place. The occurrence of opines in infected wound sites is the actual evidence for the transformation of plant cells. Considering this, experiments were conducted to find out the effect of

FIGURE 1.10 CROWN GALL TUMOR ON TOMATO PLANT
(ONE MONTH AFTER INOCULATION
WITH *A. TUMEFACIENS*)



FIG. 1.10

different incubation periods on the occurrence of opine. In these experiments, 2 months old sugarcane plants were inoculated with A. tumefaciens strains 2146, 2147, 2148, 2232 and 2822 as described earlier. 15-20 μ l of the extract, obtained from stem segments of sugarcane plants which were inoculated with 5 different strains of A. tumefaciens for 8-30 days period, was spotted on Whatman 3 paper and electrophoresis was done as described earlier. The extract of tomato tumor tissue served as a positive standard (Fig. 1.11). It can be seen from Fig. 1.11 that extract of tomato tumor tissue gives a spot corresponding to standard octopine while extract of healthy and uninoculated tomato stem tissue does not show the presence of octopine. However, in case of sugarcane, there is an absence of octopine under similar experimental conditions.

FIGURE 1.11 ELECTROPHORETIC ANALYSIS FOR
OCTOPINE DETECTION

Lanes 1 and 5	Octopine (O), arginine (A) and nopaline (N) standards
Lane 2	Extract of sugarcane shoot apex inoculated with <u>A. tumefaciens</u>
Lane 3	Extract of crown gall tissue from tomato plant (positive control)
Lane 4	Extract of tomato stem explants (negative control)

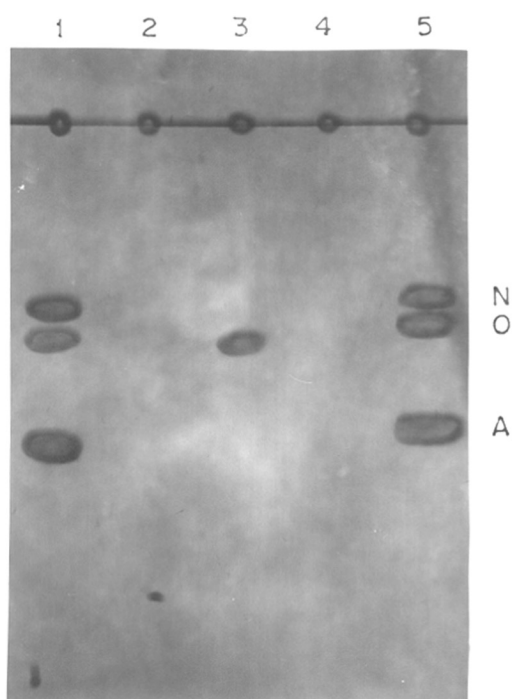


FIG. 1.11.

DISCUSSION

In this Chapter, I have described a method for rapid multiplication of sugarcane using shoot tips. To my knowledge, this is the first report of rapid multiplication of sugarcane using stem tip culture. Earlier, Sauvaire and Galzy (1978) had used axillary buds of sugarcane for the vegetative propagation of sugarcane. However, no mention was made of the number of plants that could be obtained on subculture or of survival and growth of plants in the field.

The second important aspect of the present work is its actual field application. In my work, I have indicated how this method can be used successfully for the growth of sugarcane plants from test tubes to the field. For example, on the basis of the rate of multiplication at each subculture (Table 1.7), it is possible to produce nearly 260,000 sugarcane shoots from a single shoot tip in four months after the first subculture. If all these shoots were kept in rooting medium, 200,000 rooted plantlets could be obtained in 15 days assuming that only 80% of the shoots rooted. These rooted plantlets can then be transferred to pots containing a mixture of soil and

TABLE 1.7: SCALE UP FOR PRODUCING 200,000 SUGARCANE PLANTS

Step No.	No. of shoots inoculated	Rate of multiplication	No. of shoots recovered	Period from beginning
1	1	1:1	1	15 days
2	1	1:1	1	1 month
3	1	1:4	4	1 month, 15 days
4	4	1:4	16	2 months
5	16	1:9	144	2 months, 15 days
6	144	1:10	1440	3 months
7	1440	1:12	17,280	3 months, 15 days
8	17,280	1:15	259,200	4 months

vermiculite for hardening which takes about 2-4 weeks. Thus about 200,000 plantlets can be obtained in 6 months. These plantlets are sufficient for planting in 10 hectare land at a population density of 20,000 plants per hectare. From Table 1.6, it can be seen that the sugarcane plants produced by in vitro propagation show very little variations in parameters like height, weight and number of canes thus produced. Thus this method could be highly valuable in sugarcane breeding programmes for rapid multiplication of any newly released varieties which could take several years using conventional methods.

Our preliminary attempts to transfer T -DNA from A. tumefaciens to sugarcane did not meet with success. Neither tumor formation nor the presence of opine could be detected. In monocots, tumor formation has been demonstrated so far only in Asparagus officinalis (Hernalsteens et al., 1984). In other monocots, expression of opine genes has been found in infected wound sites of Chlorophytum capense (Liliaceae), Narcissus cv. Paperwhite (Amaryllidaceae) (Hooykaas et al., 1984), Dioscorea bulbifera (Schafer et al., 1987) and Zea mays (Gramineae) (Graves and Goldman, 1986).

The inability of Agrobacterium to induce tumors on monocots or alternatively the inability of monocots to respond to infection by forming tumors has not been explained satisfactorily (DeCleene and Deley, 1976). It has been suggested that no T-DNA transfer occurs either because the bacteria cannot attach to the cell walls of monocots (Rao et al., 1982) or because of an abnormal auxin-cytokinin balance in monocot cells (Krens et al., 1983). It is also possible that the transformation frequency in monocots may be so low that it does not result in symptom formation. An alternative explanation for the failure of Agrobacterium to transform monocots is that there might be insufficient amounts of specific wound substances produced in these plants which could induce the *vir* regulon, a set of trans-acting sequences of the Ti plasmid involved in T-DNA excision and transfer (Stachel et al., 1986). Recently Chen et al., 1987) have demonstrated the transformation of sugarcane protoplasts by direct uptake of a selectable chimaeric gene but their attempts of transformation of sugarcane protoplasts by co-cultivation with Agrobacterium carrying chimaeric genes failed as sugarcane protoplasts did not survive co-cultivation procedure. The recently available information on transformation of Dioscorea bulbifera by Schafer et al. (1987) is very encouraging.

According to them, for successful tumorigenesis, induction of the vir regulon seems to be one of the pre-requisites which in their experiments is apparently brought about by treatment of bacteria with wound exudates from dicotyledonous plants. In our future plans, we wish to carry out transformation of sugarcane using various other techniques that are currently available. Our main emphasis will be on genetic modification of sugarcane to obtain disease resistant plants.

The last important outcome of the present work is the identification of an interesting differentiating sugarcane system which can be further studied using different molecular approaches. In this system, CO-740 callus exhibits a differentiating capacity over 100 subcultures whereas CO-7219 callus loses its differentiating capacity after 10 subcultures. The shoots formed by CO-740 callus is a differentiated system while CO-740 callus and CO-7219 callus cultures are dedifferentiated systems. The molecular studies on this system formulate the remaining part of the present thesis.

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CHAPTER II

ASSESSMENT OF CHANGES IN NATIVE SUGARCANE DNA IN DIFFERENTIATED AND DEDIFFERENTIATED TISSUES

SUMMARY

Physicochemical characterization of native sugarcane DNAs was carried out to assess some gross molecular changes during in vitro shoot differentiation. These studies included determination of certain properties of DNA such as thermal denaturation, buoyant density in CsCl, content of 5-methyl cytosine and pattern of distribution of methylated sequences in total genomic DNA.

The melting temperature of the three sugarcane DNAs from CO-740 callus, CO-7219 callus and CO-740 shoot were in the range of 86.2° to 86.5°C. The first derivative melting profiles were polyphasic and indicated skewing on G + C region. The DNAs from the two callus tissues banded as a single narrow peak with a buoyant density of 1.702 g/cm³ while that from CO-740 shoot banded with a buoyant density of 1.703 g/cm³. CO-740 shoot DNA was more digested with Bam HI, EcoRI, AluI and Hind III as compared with the digestion of two callus DNAs. The three sugarcane DNAs did not show any organised repeat structure with these enzymes. HpaII digested all the three sugarcane DNAs to a lesser extent than the digestion with MspI indicating less methylation of 5'-CCGG-3' sequences. The 5-mC proportion was 4 moles percent for CO-740 callus and CO-740 shoot DNAs; and 9 moles percent in CO-7219 callus DNA. The three sugarcane DNAs thus indicated similarities in melting temperature and buoyant density; and a few changes with respect to digestion with Bam HI, EcoRI, AluI and Hind III and content of 5-mC.

INTRODUCTION

Molecular studies in tissues during development have clearly shown that the differences among specialised cells are due to a differential gene activity. For example, using various nucleic acid hybridization techniques, extensive changes in mRNA populations during differentiation have been reported in many different animal cell types (Ryffel and McCarthy 1975; Hastie and Bishop 1976; Galau et al 1976; Affara et al. 1977; Blumberg and Lodish 1980; Sheperd and Nemer 1980; Jacquet et al. 1981).

In plants, variation in gene expression during cell development has been studied only recently (Kamalay and Goldberg 1980; Galau and Dure 1981; Goldberg et al. 1981; Vaillant 1983). For example, the work on DNA characterization to understand the molecular basis of cell differentiation has been carried out mainly in leaf and fruit in Cucumis melo and C. sativus (Pearson et al 1974), suspensor cells in Tropaeolum (Nagl 1976; Nagl et al 1976), epicotyl cells in pea (Van Oostveldt and Van Parijs 1976) and also during phase change from juvenile to adult in ivy (Schaffner and Nagl 1979). These studies have indicated that most of the cells in higher plants do have quantitatively different DNAs in their nuclei (Nagl 1978) in different phases of growth.

In the previous chapter, we have mentioned about the identification of in vitro differentiating system in

sugarcane. In this system, CO-740 callus represents mainly undifferentiated mass of cells while CO-740 shoot is the differentiated tissue. CO-7219 callus is different from CO-740 callus since it loses its ability to differentiate after 7-10 subcultures. We were, therefore, interested in assessing the molecular basis of differentiation in this system. As a first step towards this direction, the DNAs from these three tissues were isolated and characterized with respect to melting temperature, buoyant density in neutral CsCl, methylation status and content of 5 methyl cytosine. This chapter contains the data obtained using the above approaches.

MATERIALS AND METHODS

Chemicals

All the chemicals used throughout the work were of Analytical Reagent (AR) or Guaranteed Reagent (GR) grade. They were obtained from British Drug House (India), E. Merck (India) or SD's (India).

Chloroform and ethanol (Swastik Laboratories Pvt. Ltd., Pune), methanol (Loba Chemie Industrial Co., Bombay) and phenol (SD's Lab. Chem Industry, Bombay) were freshly distilled prior to their use. Other chemicals like sodium dodecyl sulfate (SDS), Trizma base, standard purines and pyrimidines like adenine (A), guanine (G), thymine (T), cytosine (C) and 5-methyl cytosine (5-mC), agarose, calf thymus DNA, and bovine pancreatic ribonuclease A were obtained from Sigma Chemical Co., USA. All restriction endonucleases were obtained either from Bethesda Research Laboratories (BRL), USA or New England Biolabs (NEB), USA. DNA molecular weight marker (λ DNA digested with Hind III) was obtained from Bio-Rad Laboratories, USA or New England Biolabs., USA.

DNA isolation

The methods of isolation and maintenance of sugarcane tissues have already been described in Chapter I.

For DNA isolation, it was always advantageous to use tissues in the exponential growth phase. Callus tissue of 21 day old and multiple shoots of 15 day old were,

therefore, collected, frozen in liquid nitrogen and stored at -70.0°C till further use. Bendich and Bolton's method (1967) was used with modification for DNA extraction. The frozen tissue was powdered in a mortar using liquid nitrogen and the finely powdered tissue was homogenised with a buffer [1% SDS, 0.1 M disodium EDTA, and 3 x SSC (SSC - 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0)] (1 ml buffer/g of tissue) in a Remi Mixer at a high speed for 1 min. Thick paste of the tissue was transferred to a conical flask and mixed with equal volume of chloroform-isoamyl alcohol mixture (24:1, v/v). The mixture was quickly shaken for 1 min and centrifuged for 1 min at 5000 RPM. The aqueous layer containing DNA was incubated at 62°C for 30 min in a water bath and then cooled to room temperature. Sodium perchlorate was added to this aqueous layer to a final concentration of 1 M and the mixture was shaken with equal volume of chloroform - isoamyl alcohol mixture (24:1, v/v) on a rotary shaker for 30 min. The aqueous layer was collected by centrifugation and was repeatedly subjected to chloroform - isoamyl alcohol treatment till there was no protein interphase. DNA was precipitated from the aqueous layer by addition of two volumes of ethanol and chilling the solution at -20°C for 2 h. The DNA fibres were removed, washed with 70% ethanol, dried and dissolved in 1 x SSC. The DNA solution was incubated with RNase A

(50 $\mu\text{g/ml}$) at 30°C for 1 h to remove the contaminating RNA. Prior to use, RNase was heated to 80°C for 10 min to make it free from DNase activity. The solution was then deprotenized with chloroform-isoamyl alcohol mixture (24:1, v/v) and DNA was precipitated as mentioned earlier.

This DNA was either stored at -20°C in ethanol as a precipitate or was dissolved in a suitable buffer and stored at 4°C. For thermal denaturation studies and for CsCl centrifugation, the DNA was dissolved in 0.12 M sodium phosphate buffer (pH 6.8). For HPLC analysis, the DNA precipitate itself was used after drying free of all ethanolic traces.

E. coli B (NCIM No.2089) and Micrococcus lysodeikticus (NCIM No.2437) were grown in a nutrient broth (1% peptone, 1% beef extract and 0.5% sodium chloride, pH 6.5 - 7.0) till $A_{600\text{ nm}}$ reached 0.8. The cells were harvested and DNA was isolated essentially according to Marmur (1961) except that for M. lysodeikticus, the cells were treated with lysozyme prior to SDS lysis at 62°C.

Criteria of purity of DNAs

(i) UV absorption spectrum: Wave length scanning of sugarcane DNAs was carried out in the range of 220 to 300 nm on a Shimadzu double beam spectrophotometer, Model UV 210A. Only those DNA preparations with an optical density ratio $A_{280}/A_{260} = 0.55$ and $A_{230}/A_{260} = 0.45$ and an absorbance less

than 0.1 at 300 nm were further used. Figure 2.1 depicts the UV profile of purified sugarcane DNAs of all the three tissues.

(ii) Nativity of DNAs

In order to assess the extent of double strandedness (nativity) of the isolated DNA, hydroxyapatite column chromatography was used. Hydroxyapatite was prepared according to Tiselius et al. (1956). Approximately 400 µg of DNA in 0.12 M sodium phosphate buffer (pH 6.8) was loaded on the hydroxyapatite column (17 x 2.5 cm) which was pre-equilibrated in the same buffer. The denatured DNA was eluted with 0.12 M sodium phosphate buffer (pH 6.8) while native duplex DNA was eluted with 0.4 M sodium phosphate buffer (pH 6.8).

DNA preparations which were more than 95% in duplex form were considered as native and were used for further characterization.

(iii) Hyperchromicity

The purified DNA preparations showed a hyperchromicity of approximately 20% after denaturation. Sharp melting profiles of DNA and absence of a foot or shallow absorbance rise before the actual start of DNA melting (Fig. 2.2) indicated the absence of single stranded DNA and RNA. Only those DNA preparations which exhibited sharp

FIGURE 2.1 UV ABSORPTION SPECTRUM OF SUGARCANE DNAs

The spectra were obtained in the wave length range of 220-320 nm. The DNAs were dissolved in T₁₀ buffer (10 mM Tris-HCl, pH 7.5)

- a. Blank T₁₀ buffer
- b. CO-740 callus DNA
- c. CO-740 shoot DNA
- d. CO-7219 callus DNA

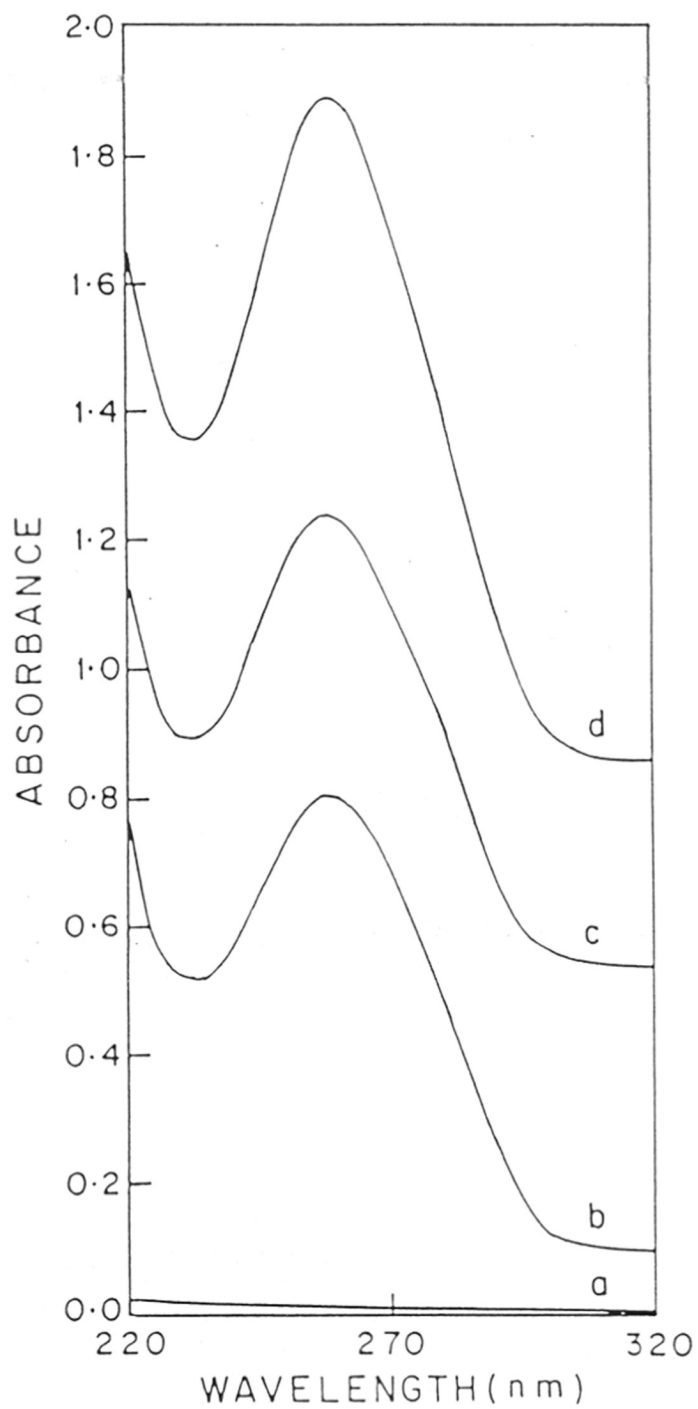


FIG. 2.1.

FIGURE 2.2 MELTING PROFILES OF NATIVE SUGARCANE

DNAs (10 kbp)

The melting of DNA was carried out in
0.12 M sodium phosphate buffer (pH 6.8)
at a heating rate of 1.0°C/min.

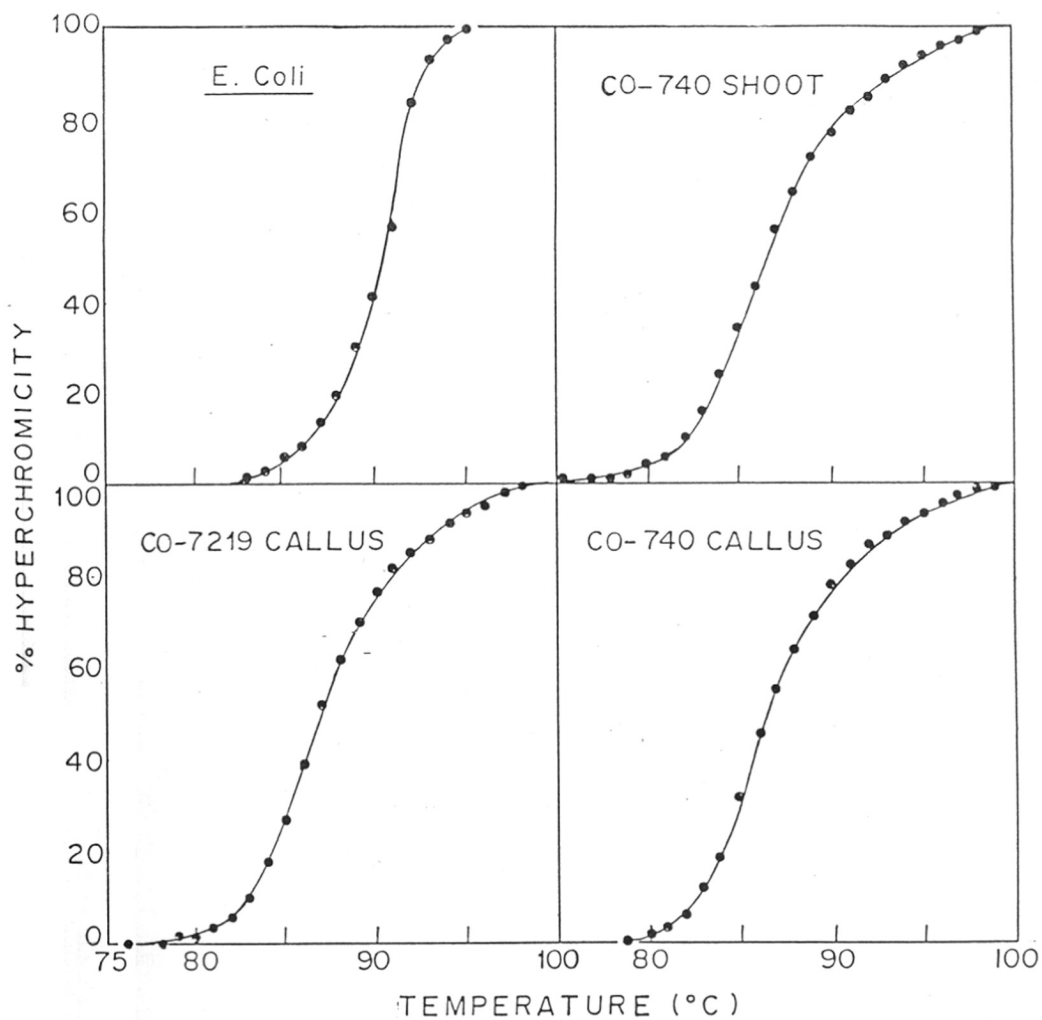


FIG. 2.2.

melting profiles and hyperchromicities greater than 20% were used for further work.

Thermal denaturation

Thermal stability of a DNA gives information about its melting temperature from which the G + C content can be calculated. The most convenient and reliable method for studying the melting of DNA is to monitor the change in its UV absorbance as a function of increasing temperature under precise environmental conditions.

Thermal denaturation of DNA was carried out in a Gilford 250 spectrophotometer equipped with thermo programmer (Model 2527), analog multiplexer (Model 6046) and automatic reference compensator (Ranjekar *et al.* 1976). Thermal cuvettes were filled with 0.3 ml of DNA solution (25-300 $\mu\text{g/ml}$) in a 0.12 M sodium phosphate buffer (pH 6.8) and the absorbance was recorded at room temperature. The cuvettes were filled in such a way that the air space was less than 10% of the solution volume. The evaporation of DNA was prevented by using teflon stoppers. The temperature of the cuvettes containing DNA solution was then raised to 98°C at a heating rate of 1°C/min. The absorbance change which occurred during the heating process was monitored with a recorder (Model 6051).

Since absorbance by nucleotide bases in the wave length region around 260 nm is due to $\pi-\pi^*$ electronic

transmission in purine and pyrimidine bases, the intensity of absorbance increases by 20-40% when the hydrogen bonds between the bases are disrupted. A linear relationship exists between this hyperchromicity and the extent of disruption of base pairs. The hyperchromicity of the DNA sample was calculated using the formula:

$$H = \frac{A_{260} \text{ at } 98^{\circ}\text{C} - A_{260} \text{ at } 62^{\circ}\text{C}}{A_{260} \text{ at } 98^{\circ}\text{C}}$$

(Zimmerman and Goldberg, 1977)

(Wimpee and Rawson, 1979)

where H = Hyperchromicity

A_{260} = Absorbance at 260 nm.

The total hyperchromicity of DNA was normalised to 100% and the graph of % hyperchromicity vs temperature was plotted. The melting temperature (T_m) of the DNA was the temperature at which half of the total increase in absorbance was obtained. The G + C content of the DNAs was determined by the formula

$$G + C = (T_m - 69.3) \times 2.44 \text{ (Marmur and Doty, 1962)}$$

The transition interval (ΔT) was calculated as

$$T_a^b = T_b^{OC} \text{ (83\% hyperchromicity)} - T_a^{OC} \text{ (17\% hyperchromicity)}$$

(Mahler and Dutton, 1964)

(Huguet and Jouanin, 1972)

DNA from E. coli was used as standard in these experiments.

High resolution thermal denaturation

Thermal denaturation at a heating rate of $1^{\circ}\text{C}/\text{min}$ displays only the overall denaturation pattern of DNA. To enhance the resolution, fine melting or high resolution thermal denaturation (HRTD) analysis was carried out using the heating rate of $0.25^{\circ}\text{C}/\text{min}$. Such analysis not only tells us about the overall distribution of bases in DNA but also helps to identify discretely melting components which are considered to represent repetitive sequence families at least in case of higher plants and animal DNAs. For high resolution thermal denaturation analysis, the sugarcane DNAs were denatured at a heating rate of $0.25^{\circ}\text{C}/\text{min}$ and absorbance values were recorded at 0.1°C increment from 62°C to 98°C for native DNA. Thus the melting curve consisted of 300-400 experimental data points depending on the melting range of DNA. A large slit width (1 mm) and a high initial DNA concentration (1-1.2 O.D. at 260 nm) helped in reducing the noise levels. Data smoothening and differentiation were performed by computer fitting of ten continuous data points at a time to a simple polynomial by the least squares method. A linear function was used as a fitting polynomial (Wada et al., 1980). The differential curves were obtained by plotting the temperature derivative of the absorbance of DNA against temperature (dA/dT vs T). The reproducibility

of the melting data was checked by using at least five DNA samples each in duplicate for melting and the resulting curves were analysed separately. The limit of resolution of this analysis was 0.001 absorbance and peaks separated by an interval as narrow as 0.4°C (approximately 1% G + C) could be detected reproducibly.

Isopycnic centrifugation in CsCl

CsCl density gradient centrifugation of DNA serves as an extremely powerful tool in differentiating the base composition of DNA. Thus DNA sequences that are enriched in G + C or A + T regions can be readily separated and purified from the bulk DNA.

CsCl density gradient centrifugation of DNA was performed using the procedure of Robkin et al., (1953). CsCl solution of initial density of about 1.700 g/ml was prepared by dissolving 951.8 mg of CsCl, 8-10 μg of sample DNA and about 5 μg of Micrococcus lysoderkticus DNA in 1 ml of SSC (X). The initial density of CsCl was calculated by measuring the refractive index of the solution using Abbe refractometer. This solution (0.4 ml) was loaded in an analytical cell and was spun in a Beckman Spinco (Model E) analytical ultracentrifuge at 36,000 RPM using ANF Ti rotor. Scans were taken at 270 nm at regular intervals of time. Attainment of density equilibrium was

confirmed by the fact that in two consecutive scans with an interval of 1 h, the DNA band had the same width and height. The density equilibrium was normally reached after centrifugation of DNA for 24-26 h. The buoyant density of DNA was calculated using the following equation.

$$\rho = \rho_0 + 4.2 \omega^2 (r^2 - r_0^2) \times 10^{-10}$$

Where ρ is the buoyant density of sample DNA, ρ_0 is the buoyant density of the marker DNA, ω is the angular velocity, r and r_0 are the radial distances of the sample and the marker DNA respectively from the axis of rotation. The buoyant density of the DNA was used to calculate G + C content of DNA using the relationship developed by Schildkraut et al. (1962).

$$G + C \% = \frac{\rho - 1.660 \times 100}{0.098}$$

where ρ is the buoyant density of the sample DNA

Sizing of DNA by agarose gel electrophoresis

The size of the native DNA was measured by agarose gel electrophoresis in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid and 2 mM disodium EDTA, pH 7.8). The DNA solution was loaded in submerged agarose slab gels (1%) and the electrophoresis was carried out at a constant current (20 mA) for 6 h. Following electrophoresis, the gels were stained in

ethidium bromide (1 µg/ml) in 1 x TAE and visualized on 302 nm long wavelength U V transilluminator (Ultra Violet Products, San Gabriel, USA). Hind III digest of DNA was used as the DNA size marker and the photographs were taken using the Minolta X 700 (35 mm) camera with red filter. The isolated sugarcane DNAs were mostly heterogeneous in size. They showed a main band of approximately 10 kbp along with a trail in the size range of 0.5 to 10 kbp.

Elution of high molecular weight DNA from agarose gels

For restriction enzyme analysis, it was essential to have a high molecular weight sugarcane DNA. A modified method of Dharmalingam (1985) involving direct elution of high molecular weight DNA from agarose gel was, therefore used. Briefly, 11 cm x 10 cm x 1 cm slabs of 0.7% agarose in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 7.8 to 8.1) were prepared. The edges of the glass plates were lined with PVC tapes to form a trough. While allowing the gel to set, four preparative (1.2 x 0.15 x 0.7 cm) slots were formed using an acrylic slot former. In the preparative slots, 600-700 µg of DNA was loaded while 2-4 µg of DNA was used in the analytical slot. The electrophoresis was carried out in 1 x TAE buffer at a constant current of 20 mA for 15-16 h. After electrophoresis, the analytical track was cut off from the slab,

stained with ethidium bromide at 1 $\mu\text{g}/\text{ml}$ concentration for 30 min and visualised on a transilluminator. The rest of the preparative slab was aligned with the analytical slab and the tracks were cut across the region corresponding to the high molecular weight DNA in the analytical track. These agarose pieces were transferred to a Sorvall SE 12 tube, submerged in phenol equilibrated with Tris buffer and frozen at -70°C for 2 h. Subsequent to this, frozen gel pieces and phenol were thawed out gradually to $4-10^{\circ}\text{C}$ and centrifuged at 10,000 RPM for 20 min at 4°C using Sorvall SE12 rotor. The aqueous layer was collected and freed of traces of phenol by treatment with chloroform - isoamyl alcohol mixture (24:1, v/v). Prior to DNA precipitation with 2 volumes of chilled ethanol, the sodium ion concentration of the aqueous solution was adjusted in equivalent to that of 0.25 M sodium acetate (pH 5.5). The DNA was allowed to precipitate either overnight at -20°C or for 20 min. at -70°C . The DNA precipitate was collected by centrifugation, washed once with 70% ethanol before drying and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Restriction endonuclease digestions and agarose gel electrophoresis

Because of site specific cleavage of DNA, the restriction endonucleases have proved to be extremely useful in studying the overall distribution of base composition of total DNA, in identification of repeated sequence families and in detecting specific methylated sequences (Fuch and Blakesley 1983; Watson and Thompson, 1986).

For all digestions, 3-5 µg of DNA was incubated with 6-10 units of enzyme at 37°C for 15-18 h in appropriate buffers (Maniatis et al., 1982). The enzyme - DNA ratio and enzyme volume to reaction volume ratio were carefully adjusted to avoid non-specific activity of the enzymes. Following incubation, the enzyme digestion was stopped by addition of 1/10th volume of a 10 x reaction stop solution (50% glycerol, 10 mM EDTA and 0.25% bromophenol blue) and digests were analysed by agarose gel electrophoresis on 1% or 1.4% neutral agarose gels in (IX) TAE buffer, pH 7.8 at a constant current of 20 mA for 5-6 h. The gels were then stained, visualized and photographed as described earlier.

High performance liquid chromatography

This is an extremely powerful and sensitive technique for analysis of various nucleic acid constituents. Among

the various HPLC types, reversed phase (RP) and ion pairing chromatography (IPC) have recently been used with considerable success in quantitation of modified bases in DNA hydrolysates as well as normal bases in body fluids as markers for disorders.

In the HPLC experiments, DNAs were hydrolysed with 60-70% perchloric acid for 1 h at 100°C. Following hydrolysis, perchloric acid was neutralised by 10 N potassium hydroxide and the potassium perchlorate precipitate was centrifuged out. Aliquots of the neutralised hydrolysate were applied to the reverse phase RP-18 column (10 μ m, HP No.79916B, 250 x 4.6 mm with a 2 μ m frit pore). The column was pre-equilibrated in 0.4 M ammonium phosphate buffer (pH 4.3) containing 5% methanol. The chromatography was carried out on Hewlett-Packard HPLC system (Model 1082B) interfaced with a Hewlett-Packard LC terminal (No.79850B) and equipped with an automatic sample injection system. The UV detector was fixed at 254 nm wave length. The chromatographic profiles were obtained at a chart speed of 1 cm/min and a flow rate of 0.5 ml/min. The elution was isocratic in the above pre-equilibrium buffer. The instrument was pre-calibrated with authentic bases namely adenine, guanine, thymine, cytosine and 5-methyl cytosine prior to injection of DNA hydrolysates. The identification of bases was

determined in terms of retention times and in case of 5-methyl cytosine, by peak enhancement due to co-injected authentic sample. The amount of 5-methyl cytosine was determined from the area of the peak in the above profiles.

RESULTS

Method of DNA extraction and yield

To assess the most suitable method for DNA isolation, three different methods namely, Bendich and Bolton's method (1967) with modifications as described in 'MATERIALS AND METHODS', Batchman and Price's method (1977) and Marmur's method (1961) were employed. The criteria of the best method was yield and hyperchromicity of the DNA. In this respect, Bendich and Bolton's method with modification was found to be the most effective and gave good DNA yield (Table 2.1). As the tissue was grown under aseptic conditions, the possibility of microbial contamination could be eliminated. Moreover, tissues with microbial contamination, if any, were discarded.

Thermal denaturation of native DNA (1°C/min.)

A DNA melting curve indicates one of the simplest forms of transition from a double helical configuration to a distorted coil (Marmur and Doty, 1959). It is obtained by plotting increase in absorbance of DNA at 260 nm (hyperchromicity) against temperature and is characterized by three main features: (i) T_m - which is the temperature corresponding to mid point of absorbance rise (Marmur

TABLE 2.1 METHOD OF EXTRACTION OF DNA, IT'S YIELD AND
HYPERCHROMICITY

Method	DNA(mg)/100 g of fresh tissue	Hyperchromicity %
Batchman and Price (3)	847 ± 78	23.24 ± 1.49
Marmur (3)	330 ± 85	15.56 ± 1.19
Bendich and Bolton (3)	1566 ± 58	23.45 ± 2.40

Figure in parenthesis indicates the number of experiments performed.

The hyperchromicity of the DNA samples was calculated using the following formula

$$H = \frac{A_{260}(100^{\circ}\text{C}) - A_{260}(62^{\circ}\text{C})}{A_{260}(100^{\circ}\text{C})} \quad \begin{array}{l} \text{(Zimmerman and Goldberg, 1977)} \\ \text{(Wimpee and Rawson, 1979)} \end{array}$$

where H is the hyperchromicity and A_{260} is the absorbance of the DNA sample at 260 nm. The total hyperchromicity was normalised to 100%.

and Doty, 1962). It is linearly dependent on G + C content of DNA and higher G + C content confers higher thermal stability of DNA; (ii) ΔT - which indicates the sharpness or the breadth of DNA melting (Mahler and Dutton, 1964; Huguet and Jouanin, 1972) and (iii) hyperchromicity - which represents the total increase in absorbance of DNA at 260 nm after complete denaturation (Zimmerman and Goldberg, 1977; Wimpee and Rawson, 1979).

Figure 2.2 depicts the melting curves of E. coli DNA, calf thymus DNA and high molecular weight DNA (10 kbp) of sugarcane CO-740 callus, CO-740 shoot and CO-7219 callus. The DNAs in these experiments were heated at the rate of 1°C/min. As it is clear from Fig. 2.2, the melting curves of E. coli DNA and sugarcane DNAs are smooth and monophasic. The melting data obtained by analyzing such curves are summarized in Table 2.2. The melting parameters such as T_m , G + C content and transition width of E. coli DNA compare well with the corresponding reported values (Blake and Lefoley, 1978). In case of three sugarcane DNAs, these three parameters vary in a narrow range of 86.2° - 86.5°C, 41.2% - 41.9% and 7.9° - 8.3°C respectively.

TABLE 2.2 THERMAL DENATURATION PROPERTIES AND BUOYANT DENSITY OF NATIVE SUGARCANE DNAs

Species	T _m (°C) ^a	G + C content ^b %	Hyper- chromicity ^c %	Δ ^{2/3} (°C) ^d	Buoyant density g/cm ³	G + C content ^e
<u>E. coli</u>	90.8 ± 0.96	52.54	21.30 ± 0.81	4.6 ± 2.31	-	-
CO-740 callus (8)	86.5 ± 0.24	41.96	23.49 ± 0.57	7.90 ± 0.12	1.702	42.85
CO- shoot (8)	86.2 ± 0.30	41.23	24.01 ± 1.72	8.32 ± 0.18	1.703	43.87
CO-7219 callus (8)	86.5 ± 0.08	41.96	23.89 ± 0.73	7.95 ± 0.17	1.702	42.85

Figure in parenthesis indicates the number of thermal denaturation experiments performed

^aTemperature that corresponds to 50% hyperchromicity

^bG + C content has been calculated using the formula: G + C content = (T_m - 69.3) x 2.44

^cCalculated using the formula: $H = \frac{A_{260}(100^\circ\text{C}) - A_{260}(62^\circ\text{C})}{A_{260}(100^\circ\text{C})}$ (Marmur and Doty, 1961)
(Zimmerman and Goldberg, 1977;
Wimpee and Rawson, 1979)

^dCalculated using the equation: Δ^{2/3} = Temperature that corresponds to 83.33% hyperchromicity -
temperature that corresponds to 16.67% hyperchromicity.

^eCalculated using the equation: % G + C content = $\frac{(\rho - 1.660)}{0.098} \times 100$ where ρ is the buoyant
density of the sample DNA

Fine melting of native sugarcane DNA (0.25°C/min)

Eukaryotic DNAs are generally very heterogeneous. However, this heterogeneity is not immediately apparent from a simple curve relating the percent hyperchromicity and temperature. It can, however, be readily detected by plotting "derived" melting curves where the ratio of hyperchromicity over a small rise in temperature (e.s. 0.1°C) to the total hyperchromicity is plotted against temperature. Such studies have revealed an ordered and non-random distribution of base sequences in most organisms.

Since fine melting analysis of DNA is a very sensitive method, several precautions were taken to avoid artefacts. Firstly, high molecular weight DNAs (10 kbp size), free from detectable levels of polysaccharides, single stranded DNA and RNA, were used. Secondly, the experimental conditions such as salt concentration (0.18 M Na⁺) and DNA concentration (50-60 µg/ml) were kept constant. About ten experiments (five preparations, each in duplicate) were carried out with the DNAs of each tissue. The number and position of all components in each preparation were reproducible in all the cases. The first derivative analysis of DNAs of E. coli and calf thymus (Fig. 2.3) which served as controls compared well with the reported values (Blake and Lefoley, 1978; Mayfield 1977, Huguet and Jouanin, 1972).

FIGURE 2.3 THE FIRST DERIVATIVE MELTING PROFILES OF
NATIVE SUGARCANE DNAs (10 kbp)

Thermal denaturation of native DNAs in
0.12 M sodium phosphate buffer (pH 6.8)
obtained with a heating rate of 0.25°C/min.
The absorbance was recorded continuously
at 0.1°C temperature intervals. The
temperature of the absorbance was
determined as described in MATERIALS AND
METHODS.

E. coli (-----) and calf thymus (—) DNAs
HRTD profiles (-) and 1°C/min heating
rate (0-0-0).

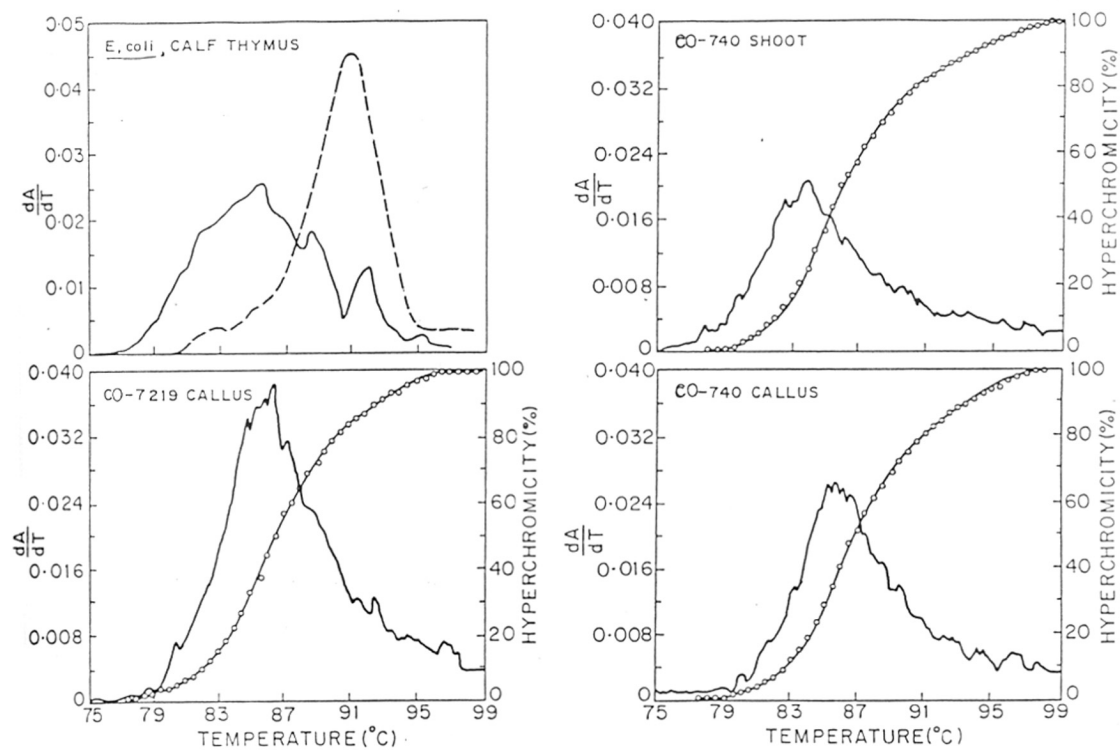


FIG. 2.3.

The high resolution derivative melting profiles of the three sugarcane DNAs are depicted in Fig. 2.3. From this figure, it can be observed that the actual melting of native DNAs starts at about 77°C and that the profiles are very heterogeneous consisting of 20-27 melting components. These melting components are divided into two groups. The first group consists of thermal components upto 86.4°C which are mostly A + T rich while the second group consists of components from 86.4° to 98°C which are mostly G + C rich. Three to four maxima peaks are present in the T_m region ($\pm 1^\circ\text{C}$ difference) in both the callus cultures. In CO-740 shoot DNA, the maxima peak does not coincide with T_m value. The high resolution derivative melting profiles of the three sugarcane DNAs consist of eight to twelve components in A + T rich region and twelve to fifteen components in G + C rich region indicating a skewing on G + C side (Table 2.3).

A qualitative comparison of the derivative melting profiles of CO-740 callus, CO-740 shoot and CO-7219 callus native sugarcane DNAs was next made in order to find out the number of components shared by them. For this purpose, a graph was plotted indicating the positions of all the melting peaks (Fig. 2.4). A temperature span of 0.2°C was considered to estimate the number of shared components and these data are summarized in Table 2.3). From Table 2.3, it can be seen

TABLE 2.3 : DISTRIBUTION OF THE COMPONENTS IN THE FINE
MELTING PROFILES OF NATIVE DNA ON EITHER SIDE
OF 86.4°C

DNA	Number of components		
	A+T rich side	G+C rich side	Total
CO-740 callus	9	15	24
CO-740 shoots	12	15	27
CO-7219 callus	8	12	20
Components shared by 3-sugarcane tissues	3	7	10
Components shared by CO-740 callus and shoot	6	8	14
Components shared by CO-740 callus and CO-7219 callus	4	8	12

FIGURE 2.4 THE POSITIONS OF MELTING COMPONENTS OBSERVED
IN THE DERIVATIVE MELTING PROFILES OF THREE
SUGARCANE DNAs

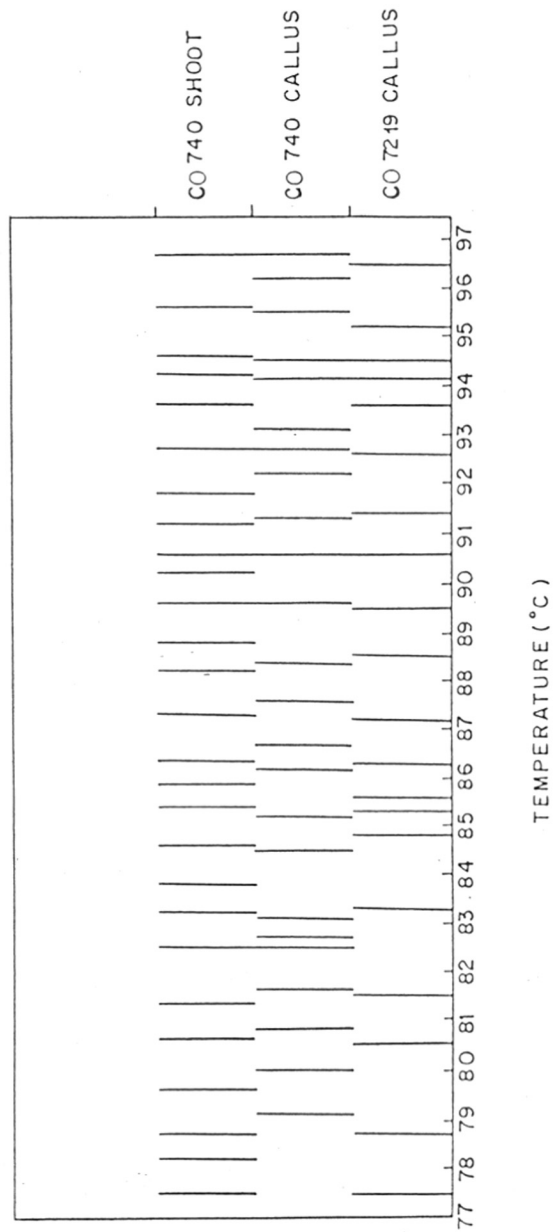


FIG. 2.4.

that in a temperature range of 0.2°C , ten components are common in all the three DNAs, three in A + T rich region and seven in G + C rich region. Among the callus cultures of CO-740 and CO-7219, four components are common in A + T rich region while eight components are common in G + C region. When CO-740 callus and shoot DNAs are compared, six components in A + T rich region and eight components in G + C rich region are common among both the cultures.

Buoyant density of DNA in CsCl

Figure 2.5 shows the CsCl profiles of the DNAs of two callus tissues namely CO-740 and CO-7219, and CO-740 shoot along with the reference DNA of Micrococcus lysodeikticus which has a buoyant density of 1.731 g/cm^3 and G + C content of 72%. The DNAs from the two calli band with a buoyant density of 1.702 g/cm^3 while that from CO-740 shoot bands with a buoyant density of 1.703 g/cm^3 . The presence of satellite DNA component is not seen in any of the three DNAs (Fig. 2.5). The G + C content is estimated from the buoyant density of each DNA and is of the order of 42.85 to 43.87% (Table 2.2). These G + C content values compare well with those obtained from the T_m data of sugarcane DNAs.

FIGURE 2.5 ANALYTICAL ULTRACENTRIFUGATION PROFILES OF SUGARCANE
DNAs IN NEUTRAL CsCl

Micrococcus lysodeikticus DNA of buoyant density
1.731 g/cm³ used as a marker. For all the profiles,
the density increase is from left to right.

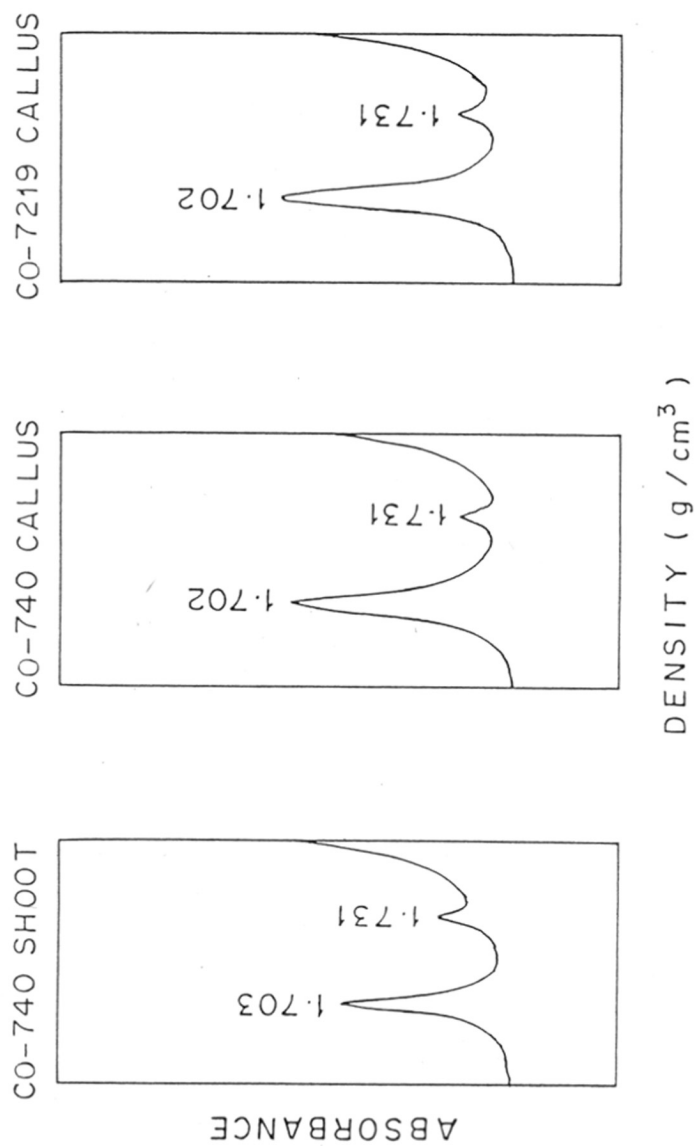


FIG. 2.5.

Behaviour of DNA towards restriction endonucleases

After determining the thermal denaturation behaviour and buoyant density values, restriction enzyme analysis of sugarcane DNAs was carried out using a few enzymes namely, AluI, Bam HI, EcoRI, Hind III, Hpa II and MspI. The objective of this work was to assess sequence specific difference in the DNAs of the three sugarcane tissues.

The digestion patterns of DNAs of CO-740 callus, CO-740 shoot and CO-7219 callus with Bam HI, Hind III, Alu I and EcoRI are depicted in Fig. 2.6. The three sugarcane DNAs are extensively digested with these four restriction endonucleases. However, some differences in the extent of their digestion are noticed. CO-740 shoot DNA, for example, is more digested with Bam HI, EcoRI, Alu I and Hind III as compared to the digestion of CO-740 callus DNA and CO-7219 callus DNA. The three sugarcane DNAs do not show any organised, repeat structure with any of the enzymes used. This suggests the occurrence of either very small spacing between successive sites for these enzymes or a random distribution of these sites.

Of the enzyme pairs that can be used to study the methylation status, the most important is an isoschizomeric pair, MspI and Hpa II, both recognising the sequence 5'-CCGG-3' but showing differential sensitivity to whether outer C or inner C is methylated or not. Msp I,

FIGURE 2.6 RESTRICTION ENZYME ANALYSIS OF SUGARCANE DNAs

CO-740 callus (Lane 2,5,8 and 11), CO-740 shoot (Lanes 3,6,9 and 12) and CO-7219 callus (Lanes 4, 7, 10 and 13) DNAs.

Lanes 2,3 and 4. Bam HI digest

Lanes 5,6 and 7. Eco RI digest

Lanes 11,12 and 13. AluI digest

Lanes 1 and 14. Hind III digest of λ DNA as a molecular weight marker (23.3 kbp, 9.5 kbp, 6.4 kbp, 4.2 kbp, 2.2 kbp and 1.8 kbp)

Electrophoresis was carried out in 1.4% agarose gel.

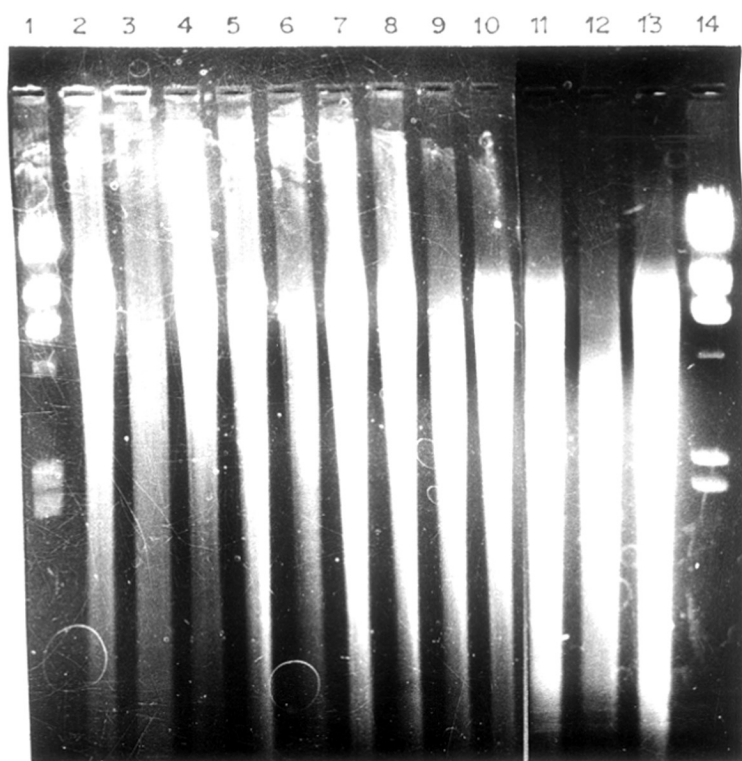


FIG. 2.6 .

for example, cleaves CmCGG but not mCCGG while Hpa II cleaves mCCGG but not CmCGG. From Fig. 2.7, it is clear that in case of all the three sugarcane DNAs, Hpa II digests the DNAs to a lesser extent than MspI. These data clearly suggest that most of the CCGG sequences are of the type CmCGG. Among the three sugarcane DNAs, CO-740 shoot DNA is more extensively digested with Msp I in contrast to CO-740 callus DNA and CO-7219 callus DNA.

Content of 5-methyl cytosine in native DNA

The restriction enzyme analysis with methylation specific enzymes gives an overall information about the methylation status of bases. The HPLC helps to detect the presence of normal as well as modified bases. The HPLC was performed using a silica banded non-polar stationary phase (hydrocarbons of various carbon chain length) and a polar mobile phase. As the stationary phase is less polar than the eluent, it is generally referred to as reversed phase chromatography. In such systems, solvophobic forces are responsible for the binding of a solute (substances to be resolved by chromatography) to the stationary phase so that polar substances elute first. Solute retention is mainly determined by solvent effects, in particular the polarity of the solvent. In order to ensure the reproducibility of the retention time of the solute, solvent

FIGURE 2.7 DIGESTION OF TOTAL SUGARCANE DNAs WITH

Msp I AND Hpa II

CO-740 callus (Lanes 2 and 5), CO-740 shoot
(Lanes 3 and 6) and CO-7219 callus (Lanes 4
and 7)

Lanes 1 and 8. Hind III digest of λ DNA as a
molecular weight marker (23.3 kbp, 9.5 kbp,
6.4 kbp, 4.2 kbp, 2.2 kbp and 1.8 kbp)

Lanes 2,3 and 4. Msp I digest

Lanes 5,6 and 7. Hpa II digest

Electrophoresis was carried out in 1.4%
agarose gel.

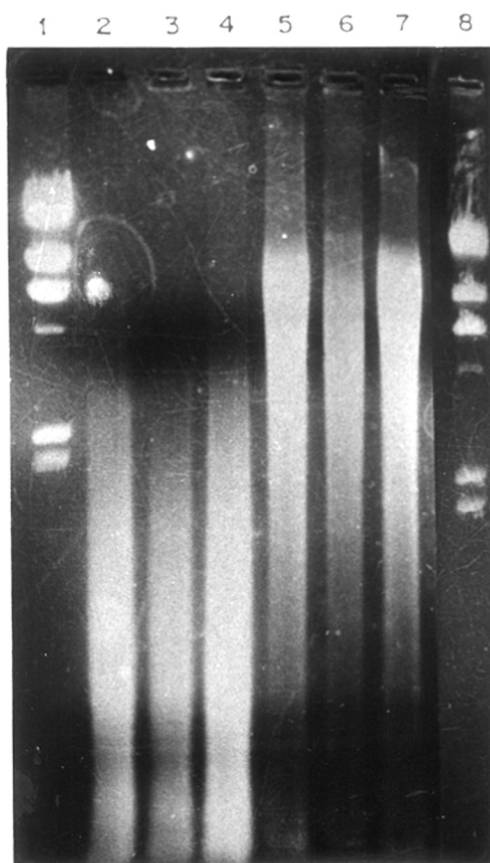


FIG. 2.7.

polarity is rigidly maintained by a critical pH adjustment and addition of specific proportions of organic solvents (Harvath, 1981).

By the use of appropriately designed instrument and micro particulate packed columns, free bases of DNA hydrolysate are identified by their retention time. The method of DNA hydrolysis employed in these studies ensures formation of all free bases so that it should be possible to detect all modified bases in addition to normal A, T, G and C.

The HPLC elution profiles of calf thymus DNA, and sugarcane DNA hydrolysates and of a standard mixture of authentic samples of adenine (A), guanine (G), thymine (T), cytosine (C) and 5-methyl cytosine (5-mC) are depicted in Fig. 2.8. The proportions of 5-mC as calculated from the area of the peak in the above profiles are given in Table 2.4. Our results show that 5-methyl cytosine content is 4% (molar basis) in CO-740 shoot and callus DNA while it is 9.6% (molar basis) in CO-7219 callus DNA.

FIGURE 2.8 HPLC ANALYSIS OF SUGARCANE DNAs

Standard mixture contains authentic bases namely cytosine (C), 5-methyl cytosine (5 mC), guanine (G), thymine (T) and adenine (A).

↓ indicates 5 mC peak.

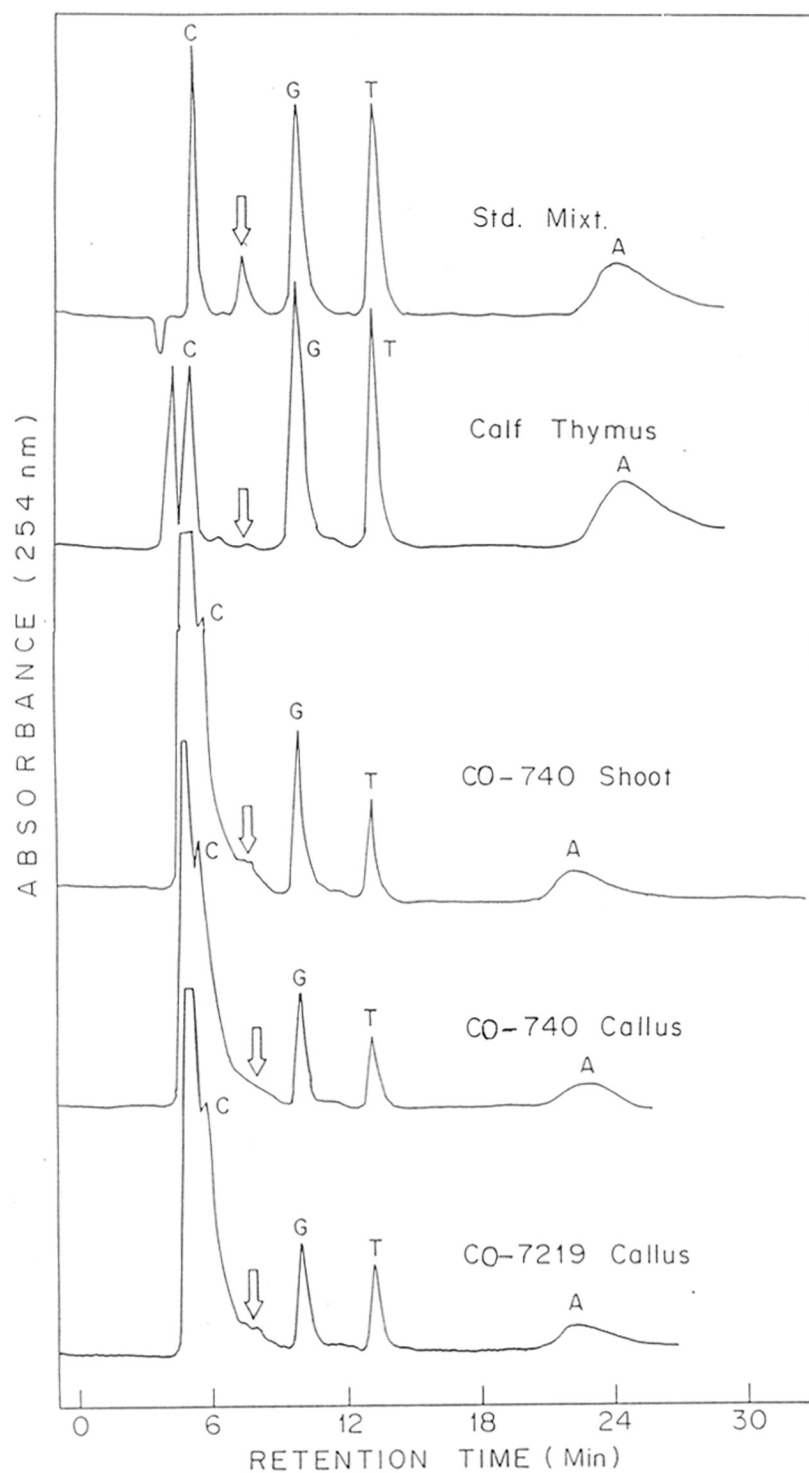


FIG. 2.8.

TABLE 2.4 PROPORTION OF 5-METHYL CYTOSINE IN NATIVE
SUGARCANE DNAs

DNA	Moles percent of [*] 5-methyl cytosine
CO-740 callus	4.2
CO-740 shoot	4.1
CO-7219 callus	9.6

* Proportions of mole percentage are calculated from peak area.

Represents an average value of 3 to 5 independent experiments.

DISCUSSION

In the present study, an attempt has been made to assess the differences at the total DNA level in three different sugarcane tissues namely, CO-740 callus, CO-740 shoot and CO-7219 callus using four independent approaches, namely, thermal denaturation at a heating rate of 1°C/min and 0.25°C/min, buoyant density determination in neutral CsCl, restriction enzyme analysis and HPLC. In the literature, scattered attempts have been made to assess DNA differences during cell differentiation. For example, thermal denaturation analysis has indicated differences in the melting temperatures of DNAs from different tissues and stages in pea epicotyl cells (Van Oostveldt and Van Parijis, 1976), developing protocorms of cymbidium (Nagl and Rucker, 1976), leaves of juvenile and adult phases in ivy (Kessler and Reches, 1977) and differentiating root systems of Allium cepa and Vicia faba (Durante et al., 1977; Bassi et al., 1984). Changes have also been observed in DNA methylation patterns of Cucumis melo (Grisvard, 1985), Vicia faba (Negruk et al., 1986) and soybean (Quemada and Lark, 1987); and in buoyant density of satellite DNA in Vicia faba (Natali et al. 1986) carrot (Schafer et al., 1978) and N. glauca (Durante et al., 1982) in differentiated and dedifferentiated tissues. Ours is probably the first effort to use as many as four approaches at a time to tackle the problem

of in vitro differentiation at the DNA level.

From Figs. 2.2 and 2.5 and Table 2.2, it is clear that thermal denaturation analysis and buoyant density measurement have not revealed any significant differences among the DNAs of CO-740 callus, CO-740 shoot and CO-7219 callus. However, some difference is seen with reference to the DNA methylation status of these three different tissues. Here, although the DNAs of all the three tissues are more digested with MspI than with HpaII, the extent of digestion with each of these two enzymes is different. For example, the digestion of CO-740 shoot DNA is significantly more than that of CO-740 callus DNA with both MspI and HpaII. Such a difference is clearly due to the variations in the number of 5'-CCGG-3' sequences in these tissues; the number being higher in shoot DNA than that in callus DNA. The second important difference among these three tissues is the content of 5-mC as revealed by HPLC measurements. The latter have indicated that CO-7219 callus DNA contains 9.6% of 5-methyl cytosine on molar basis as against 4.2% in CO-740 callus and shoot DNA. It can be seen from Table 2.5 that 5-methyl cytosine content of CO-740 shoot DNA and CO-740 callus DNA is comparable to that of other plants. CO-7219 callus DNA is rather unique in having a high proportion of 5-mC. As said earlier, CO-7219 callus tissue is different from CO-740 callus in the fact that it loses its differentiating ability

TABLE 2.5: 5-METHYL CYTOSINE CONTENT IN DIFFERENT PLANTS*

Plant	5 methyl cytosine (Mol %)
<u>Daucus carota</u>	6.0
<u>Cucurbita pepo</u>	3.7
<u>Phaseolus vulgaris</u>	5.2
<u>Gossypium hirsutum</u>	4.6
<u>Triticum</u> sp.	6.0
<u>Zea mays</u>	6.2
<u>Allium cepa</u>	5.4
<u>Cymbidium pumilum</u>	3.5
Sugarcane CO-740 shoot (Present Study)	4.1
Sugarcane CO-740 callus (Present Study)	4.2
Sugarcane CO-7219 callus (Present Study)	9.6

* J.A. Brant (1976)

after 10 subcultures. CO-740 callus, on the other hand, has retained its capacity for differentiation even after 100 subcultures. In view of its high content of 5-mC in CO-7219, it is likely that 5-mC may have some role in its inability for differentiation after specific number of subculturing. Since it is known that actively transcribing genes are generally under methylated, methylation may lead to the inactivation of certain genes in CO-7219 callus as a result of which it loses its ability to differentiate.

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CHAPTER III

ANALYSIS OF REPEATED AND SINGLE
COPY DNA SEQUENCES IN TOTAL SUGARCANE DNA
FROM CALLUS AND SHOOT TISSUES

SUMMARY

Non-linear regression analysis of reassociation curves of sonicated sugarcane DNAs from two callus cultures, namely CO-740 and CO-7219 and shoots of CO-740 revealed the presence of one fast and one slow DNA component, each reassociating with second order kinetics. A comparison of rate constants of these two DNA components indicated that the fast reassociating DNA fraction consisted of repeated DNA sequences while slow reassociating DNA fraction included mainly single copy DNA sequences. Marked differences were noticed in the amount, kinetic complexity and size distribution of repeated DNA sequences in callus and shoot tissues. In CO-740 callus, the fast DNA comprised 47.5% of the total DNA as against 16.5% in CO-740 shoot and 38.5% in CO-7219 callus. The proportion of the slow component was 25-30% in all the three DNAs. Its kinetic complexity was in the range of $2.5-4.4 \times 10^8$ nucleotide pairs in both the callus cultures and was significantly higher than that in shoot DNA (6.1×10^6 nucleotide pairs). The reassociation kinetics data thus revealed that repeated DNA sequences have undergone a significant change during differentiation of callus tissue to shoots in sugarcane.

INTRODUCTION

It is generally accepted that eukaryotic DNA is dynamic and undergoes selective rearrangements and changes during cell differentiation (Nagl and Rucker, 1976; Buiatti, 1977, Verma and Dougall, 1978; Durante et al. 1983; Ssymank, 1983 and Duhrrssen et al. 1984). For example, differential replication of satellite DNA and main band DNA has been described during dedifferentiation in Vicia faba (Natali et al. 1986), carrot (Hase et al. 1979) tobacco (Durante et al. 1983) and melon (Grisvard and Anghileri, 1980; Grisvard, 1985). The rDNA, however, has been shown to undergo no gross change during dedifferentiation in carrot and Jerusalem artichoke explants (Hase et al. 1982).

In the tissue culture chapter (chapter I), we have already described the identification of a differentiating sugarcane system. In our efforts to assess the molecular basis of differentiation, we have characterized the total DNA of sugarcane and these data are described in chapter II. In further continuation of this molecular work, we determined the reassociation kinetics of total DNA of the three sugarcane tissues namely CO-740 callus, CO-740 shoot and CO-7219 callus and these data are detailed in the present chapter.

MATERIALS AND METHODS

DNA isolation

The method of isolation of DNA from the three sugarcane tissues has already been described in Chapter II. Only those DNA preparations with an optical density ratio of $A_{280}/A_{260} = 0.55$ and $A_{230}/A_{260} = 0.45$ and an absorbance less than 0.1 at 300 nm were further used.

DNA shearing and sizing

For all the reassociation experiments described in this chapter, sonicated DNA of an average fragment size of 0.5 kbp was used. Before sonication, native sugarcane DNA was dialysed against 0.12 M sodium phosphate buffer (pH 6.8) and was fragmented to a modal length of 0.5 kbp using a Sonic Oscillator (Brownwell Model, Biosonik III, 250 W, 20 KHz) fitted with a 12 mm probe. Approximately 30 ml of DNA solution was sonicated at a maximum intensity for 3 mins with an interval of 10 min between each 1 min of sonication. Care was taken to see that the temperature of the DNA solution did not rise above 4°C.

The average size of DNAs was determined by agarose slab gel electrophoresis using 0.8 to 1% agarose gels in Tris-(hydroxymethyl)-aminomethane (Tris) (40 mM)- acetic acid (20 mM)-EDTA (2 mM) at pH 8.1. Agarose gels were stained with ethidium bromide (5 µg/ml) for 30 min and the DNA bands were photographed using 400 ASA, 35 mm negative

film after visualization on a transilluminator. Lambda phage DNA digested with Hind III served as a molecular weight marker. The DNA preparations without sonication had a trailing in the range of 10 kbp to 0.5 kbp size while sonicated DNAs had the average size of 0.5 kbp.

DNA reassociation kinetics

The two main steps in a typical DNA reassociation experiment involve DNA strand separation by heat denaturation and reassociation of complementary DNA strands. A proper choice of incubation temperature (62°C , $T_m - 25^{\circ}\text{C}$), salt concentration (0.12 M sodium phosphate buffer, pH 6.8 i.e. 0.18 M Na^+) and DNA fragment length (0.5 kbp) provides optimum conditions for DNA reassociation. In the the present work, two techniques were used to measure the rate of DNA reassociation.

(i) Optical reassociation

Since DNA strand separation produces a hyperchromic shift in absorbance which is reversed as base pairs reform, reassociation of DNA may be followed by monitoring the decrease in absorbance as a function of time. Gilford 250 spectrophotometer equipped with a thermoprogrammer (Model 2527), analogue multiplexer (Model 6046) and automatic reference compensator was used for this purpose. DNA samples, 0.5 kpb long, in 0.12 M sodium phosphate buffer (pH 6.8)

were added to thermal cuvettes (0.3 ml capacity) and temperature of the solution was raised to 100°C. The absorbance change occurring during the heating process from 62° to 100°C was monitored at 260 nm. The hyperchromicity of the DNA sample was calculated using the following formula:

$$H = \frac{A_{260}(100^{\circ}\text{C}) - A_{260}(62^{\circ}\text{C})}{A_{260}(100^{\circ}\text{C})} \quad \text{(Zimmerman and Goldberg, 1977; Wimpee and Rawson, 1979)}$$

where H is the hyperchromicity and A_{260} is the absorbance of the DNA sample at 260 nm. The total hyperchromicity was normalized to 100%. The denatured DNA samples were maintained at 100°C for 4-5 min to ensure complete denaturation and were then quickly cooled to 62°C by circulation of cold water. The change in absorbance of the DNA was continuously recorded with time. For the determination of zero time or the start of reassociation, the mid point (80°C) between 100°C and 62°C was considered (Britten et al., 1974). A Cot value at different time intervals is defined as half the product of absorbance of denatured DNA x hours of incubation i.e. $Cot = \frac{A \times t}{2}$ (h) (Britten and Kohne, 1968; Kohne and Britten, 1971). Thus a Cot value of one is equivalent to incubation of DNA solution at a concentration 83 µg/ml (approximately 2 O.D.) for 1 h. The percentage reassociation at different Cot values was calculated using the formula:

$$\% R = \frac{A_{260} \text{ of denatured DNA} - A_{260} \text{ at a given time}}{A_{260} \text{ of denatured DNA} - A_{260} \text{ of native DNA at } 62^{\circ}\text{C}} \times 100$$

The reassociation of DNA was monitored optically in the Cot range of 1×10^{-3} to 1×10^1 M.s.

(ii) Hydroxyapatite column chromatography

Hydroxyapatite was prepared essentially according to the procedure of Tiselius et al. (1956) and stored at 4°C with a few drops of chloroform. The hydroxyapatite preparations usually formed columns with good flow characteristics. The extent of DNA recovery from each batch of hydroxyapatite was checked by loading known quantities of DNA and the recovery was always more than 95%. For the preparation of hydroxyapatite columns, hydroxyapatite slurry was poured into a double jacketed glass column (15 x 2.5 cm) to a bed volume of approximately 15 ml. The column was equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) and maintained at 62°C with a thermostatic circulating waterbath. The hydroxyapatite chromatography was essentially according to Kohne and Britten (1971) and Britten et al. (1974). Sonicated DNA samples ranging in concentration from 30 $\mu\text{g/ml}$ for lower Cot values to 300 $\mu\text{g/ml}$ for higher Cot values in 0.12 M sodium phosphate buffer (pH 6.8) were sealed in boiling tubes and denatured by keeping in a water bath at 100°C for 10 min. These tubes were then incubated at specific Cot values at 62°C . The samples were

loaded on columns which were pre-equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) and maintained at 62°C. Single stranded DNA was eluted with 0.12 M sodium phosphate buffer (pH 6.8) while double stranded DNA was eluted with 0.4 M sodium phosphate buffer (pH 6.8). The relative amounts of single and double stranded DNA were determined by measuring absorbance of DNA fractions at 260 nm on a Shimadzu double beam spectrophotometer (Model UV-210A) assuming that one absorbance unit (A_{260}) is equal to 50 µg of DNA/ml. The extent of reassociation was calculated using the formula:

$$\% R = \frac{\mu\text{g of DNA eluted with 0.4 M sodium phosphate buffer (pH 6.8)}}{\text{total } \mu\text{g of DNA eluted from the HAP column}} \times 100$$

In experiments at high Cot values ($>1 \times 10^2$ Ms), the DNA samples were incubated in 0.36 M sodium phosphate buffer (pH 6.8) in order to accelerate the rate of reassociation and thus reduce the incubation time. A corresponding correction was applied to the reassociation rate using the factor 4.4778 (Britten et al. 1974). DNA samples reassociated in higher salt concentrations were diluted to 0.12 M sodium phosphate buffer (pH 6.8) with double distilled water prior to loading onto a hydroxy-apatite column.

After completing the DNA reassociation experiments at various Cot values, a graph of percent reassociation

versus Cot was plotted on a semilogarithmic paper. The data points obtained optically and by hydroxyapatite column chromatography were fitted together for the construction of the Cot curve. Reassociation kinetics of E. coli DNA and mouse liver DNA, studied under identical conditions, served as internal standards.

Non-linear least squares regression analysis of reassociation kinetics data

Eukaryotic DNAs generally reassociate over a range of Cot values spanning upto eight orders of magnitude or even more. Since the individual DNA components of the reaction are not usually well separated, a computer programme was used to generate a set of Cot curves whose sum fits the experimental data. The individual curves represent single kinetic components and are fitted to the second order equation by least square analysis of the data. These components may not exist as discrete entities in the genome but represent a simplified mathematical description of the data. The programme is usually run on the basis of finding the minimum number of curves required to fit the data (Pearson et al. 1977).

Since DNA reassociation follows second order kinetics, it can be defined using the equation $\frac{dC}{dt} = -KC^2$ where C is the concentration of single stranded sequences and t is the incubation time (Britten et al. 1974). This yields

the equation $\frac{C}{C_0} = \frac{1}{1 + KCot}$ where C_0 is the initial concentration of single stranded DNA in moles nucleotides per litre i.e. $C = C_0$ when $t = 0$. Least squares analysis of the experimental data was carried out by employing a standard optimization subroutine STEPIT (written by J.P. Chandler and distributed by QCPE, Indiana University, Bloomington, Indiana, USA). The computer fitted curves are drawn as a solid line through the data points and the dotted lines represent kinetics of the individual components if they existed alone.

The RMS error is given by the equation:

$$\text{Error} = \sum \left[\left(\frac{C}{C_0} \right)_{\text{expt.}} - \left(\frac{C}{C_0} \right)_{\text{fit}} \right] \text{--- Equation I}$$

and was minimized by a direction search procedure.

The form for $\frac{C}{C_0}$ was assumed to be

$$\frac{C}{C_0} = T + \sum \frac{F_i}{1 + K_i Cot} \text{--- Equation II}$$

where $\frac{C}{C_0}$ is the fraction denatured,

T the fraction of the genome that fails to reassociate

F_i the fraction of the genome reassociating in the i^{th} component

K_i the reassociation rate for the i^{th} component

The parameters T, F_i and K_i are allowed to free float to minimize the error in Equation I. The computer used for such curve fitting procedures was ICL 1900 series.

RESULTS

Precautions taken during reassociation kinetics studies

The 230/260 and 280/260 ratios of the isolated sugarcane DNAs were often high. This could be due to presence of proteins and/or carbohydrates. Purification of such DNA preparations by hydroxyapatite column chromatography helped to obtain DNA preparations with desired 230/260 and 280/260 ratios. In this purification procedure, the isolated DNA preparations were equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) and then loaded on HAP columns which were also pre-equilibrated with 0.12 M sodium phosphate buffer (pH 6.8) at room temperature. Single stranded DNA was eluted with 0.12 M sodium phosphate buffer (pH 6.8) while double stranded DNA was eluted with 0.4 M sodium phosphate buffer (pH 6.8).

Measurement of reassociation of DNA by hydroxyapatite column chromatography can lead to an overestimate of double stranded DNA due to the presence of single stranded tails and hyperpolymers. The use of very small fragments of DNA, therefore, can minimise this problem and provide a better estimate of the extent of reassociation at a given Cot value. Hence in the present work, DNA fragments with an average size of 0.5 kbp were used. Recovery of DNA from hydroxyapatite at this fragment length was nearly 100% indicating the absence of hyperpolymer formation.

Finally, the rate of reassociation is dependent on the concentration of the complementary DNA strands in solution, temperature of incubation and salt concentration. Extreme care was taken to maintain the proper conditions of reassociation. Typically, DNA concentrations were kept between 30-300 $\mu\text{g/ml}$ to avoid viscosity effects on the rate of reassociation (Pays and Ronsse, 1975) and the temperature and salt concentration were 62°C and 0.18 M Na^{+} , respectively.

Using the above parameters for reassociation, reassociation kinetics of E. coli and mouse liver DNA were determined and these were used as controls.

Reassociation kinetics of sugarcane DNAs

Non-linear least squares regression analysis of the reassociation data obtained by optical measurements and hydroxyapatite column chromatography helped in determining the number of components reassociating with second order kinetics in the three sugarcane DNAs. The results of such analysis are depicted in Fig. 3.1 and are summarized in Table 3.1. From these data, it can be seen that one fast and one slow DNA component, each reassociating with second order kinetics is present in all the three sugarcane tissues studied. From the $\text{Cot } \frac{1}{2}$ of fast and slow DNA fractions, it is clear that the fast DNA reassociates 6 to 726 times faster than the slow DNA. This indicates that the fast reassociating DNA fraction consists of repeated DNA

FIGURE 3.1 REASSOCIATION KINETICS OF SONICATED

SUGARCANE DNAs (0.5 kbp)

Reassociation curves of 0.5 kbp long DNA

of CO-740 callus, CO-740 shoot and CO-7219 callus

using optical spectrophotometry as well as hydroxy-

apatite column chromatography. The solid line through

the data points represents the least squares fit for the

components allowing all parameters to free float. The lower

dashed curves represent the reassociation kinetics of pure components

Mouse DNA (0-0-0) and E. coli DNA () were used as control

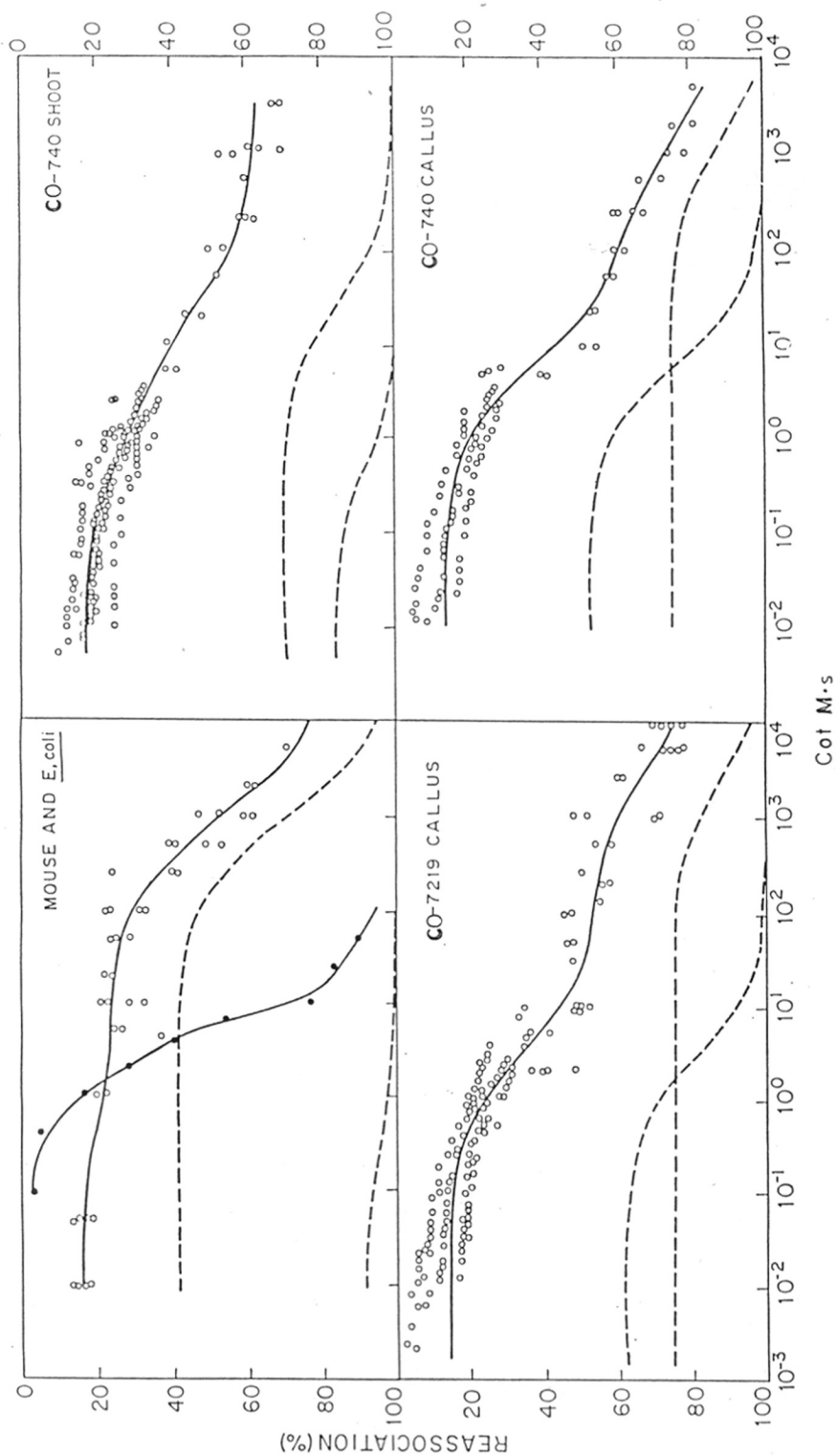


FIG. 3.1.

TABLE 3.1 REASSOCIATION KINETICS OF SUGARCANE DNAS

DNA fraction	Fraction ^a of DNA	Cot $\frac{1}{2}$ ^a observed M.s.	Frequency of repetition	Cot $\frac{1}{2}$ ^c pure M.s.	Kinetic complexity ^d nucleotide pairs
1	2	3	4	5	6
<u>CO-740 Callus</u>					
Very fast reassociating DNA (Cot $< 10^{-2}$ M.s.)	0.14	-	-	-	-
Fast reassociating DNA (Cot 1×10^{-2} to 1×10^2 M.s.)	0.47	6.40	2.09×10^2	3.04	2.28×10^6
Slow reassociating DNA (Cot 1×10^2 to 5×10^3 M.s.)	0.25	1.34×10^3	1	3.43×10^2	2.55×10^8
Unreassociated DNA (Cot $> 5 \times 10^3$ M.s.)	0.12	-	-	-	-
<u>CO-740 shoot</u>					
Very fast reassociating DNA (Cot $< 5 \times 10^{-3}$ M.s.)	0.16	-	-	-	-
Fast reassociating DNA (Cot 5×10^{-3} to 2.5 M.s.)	0.16	4.56	5.80×10^1	7.63×10^{-2}	5.70×10^4
Slow reassociating DNA (Cot 2.5 to 2×10^3 M.s.)	0.30	2.71×10^1	1	8.16	6.12×10^6
Unreassociated DNA (Cot $> 2 \times 10^3$ M.s.)	0.37	-	-	-	-

TABLE 3.1 (continued)

1	2	3	4	5	6
<u>CO-7219 Callus</u>					
Very fast reassociating DNA (Cot $< 10^{-2}$ M.s.)	0.14	-	-	-	-
Fast reassociating DNA (Cot 1×10^{-2} to 1×10^2 M.s.)	0.38	3.11	7.26×10^2	1.19	8.99×10^5
Slow reassociating DNA (Cot 10^2 to 5×10^3 M.s.)	0.25	2.26×10^3	1	5.84×10^2	4.38×10^8
Unreassociated DNA (Cot $> 5 \times 10^3$ M.s.)	0.20	-	-	-	-

^aValues obtained from the respective reassociation curves (Fig. 3.1)

^bCot 1/2 values of unique DNA divided by Cot $\frac{1}{2}$ values of the given fraction.

^cCot 1/2 values observed x fraction of genome.

^dValues obtained by comparing the Cot 1/2 of E. coli DNA under our experimental conditions and assuming the genome size of E.coli as 4.5×10^6 base pairs (Cairns, 1963).

sequences while the slow reassociating DNA fraction is made up of mainly single copy DNA sequences. Marked differences are, however, noticed among the three systems in the proportion, frequency of reiteration and kinetic complexity of the fast reassociating DNA component.

In CO-740 callus, for example, the fast DNA reassociating between $Cot\ 1 \times 10^{-2}$ - 1×10^2 Ms comprises 47.5% of the total DNA. In CO-740 shoot and CO-7219 callus on the other hand the fast DNA reassociates in the range of $Cot\ 5 \times 10^{-3}$ to 2.5 Ms and $Cot\ 1 \times 10^{-2}$ to 1×10^2 Ms respectively and represents 16.5% in CO-740 shoot and 38.5% in CO-7219 callus. The frequency of repetition and kinetic complexity of the fast DNA sequences are also high in callus cultures as compared to those in shoots. The proportion of the slow component is 25-30% in all three tissue systems. Its kinetic complexity is in the range of $2.5 - 4.4 \times 10^8$ nucleotide pairs in both the types of callus cultures and is significantly higher than that of shoot DNA (6.1×10^6 np).

Apart from the fast and slow reassociating DNA components, 14-16% of the total DNA forms duplexes very rapidly at $Cot < 10^{-2}$ Ms in all the three sugarcane tissues. These are instantaneously reassociating sequences and may consist of highly repeated DNA and/or fold back sequences (Walbot and Dure, 1976). There is 12-37% of the total

sugarcane DNA which fails to reassociate at $Cot > 10^3$ Ms. This may be because of degradation of DNA during long incubation periods, failure of DNA to form stable duplexes and/or the presence of some sequences requiring a longer time to reassociate (Cullis, 1981, Pearson et al. 1978).

DISCUSSION

With the advent of recombinant DNA technology, it has been possible to clone specific genes and characterize them in details with respect to their coding regions and regulatory sequences in the 5' upstream and 3' downstream regions. DNA reassociation kinetics, although relatively an old experimental approach, gives a general guidance to genome structure and also an overall information about the content of repetitive DNA and about average estimates of its copy number and frequency of repetition. These studies are a prerequisite for further work such as DNA sequence organization and interspecies DNA sequence homologies.

Although the genomes of several plant species have been characterized with respect to their kinetic complexity and content of repetitive DNA, no such information was available in sugarcane when this work was undertaken. The present study has shown for the first time that the content of total repetitive DNA in sugarcane shoot genome is of the order of 33%. In the family Gramineae, cereals with high DNA content ($1 \text{ C} > 5 \text{ pg}$) such as wheat, oat, rye, barely and maize have been extensively characterized with respect to their genome organization (Flavell and Smith, 1976; Hake and Walbot, 1980; Rimpau et al. 1978; 1980; Smith and Flavell, 1977). In these species, repeated DNA sequences constitute a major proportion (60-80%) of the total DNA (Table 3.2)

TABLE 3.2: GENOME SIZE AND PROPORTIONS OF REPETITIVE AND UNIQUE DNA IN GRAMINEAE

Species	Genome size 1C	Total repetitive %	Total repetitive pg	Unique DNA %	Unique DNA pg	Reference
<u>Eleusine coracana</u> (Finger millet)	1.6	49.0	0.78	51.1	0.81	Deshpande and Ranjekar (1980)
<u>Echinochloa frumentacea</u> (Barn yard millet)	2.70	42.0	1.13	62.0	1.67	SivaRaman and Ranjekar (1984)
<u>Hordeum vulgare</u> (Barley)	5.4	78.0	4.25	22.0	1.19	Ranjekar et al. (1978)
<u>Oryza sativa</u> (Rice)	0.6	52.0	0.31	48.0	0.28	Deshpande and Ranjekar (1980)
<u>Panicum miliare</u> (Little millet)	1.0	38.0	0.38	62.0	0.62	ShivaRaman and Ranjekar (1984)
<u>Pennisetum americanum</u> (Pearl millet)	2.4	54.0	1.32	46.0	1.12	Deshpande and Ranjekar (1980)
<u>Saccharum officinarum</u> (Sugarcane)	4.0	33.0	1.32	67.0	2.68	Present study
<u>Setaria italica</u> (Fox tail millet)	0.8	30.0	0.24	70.0	0.56	SivaRaman and Ranjekar (1984)
<u>Secale cereale</u> (Rye)	9.0	73.5	5.85	26.5	3.28	Ranjekar et al. (1978)
<u>Sorghum vulgare</u> (Great millet)	4.6	52.0	2.39	48.0	2.20	SivaRama et al. (1984)
<u>Triticum aestivum</u> (Wheat)	17.3	80.0	13.84	20.0	3.46	Ranjekar et al. (1976)
<u>Zea mays</u> (Maize)	7.7	58.0	4.46	42.0	3.23	Hake and Walbot (1980)

The work on millet DNAs (1C : 0.8-2.8 pg) has revealed several distinguishing features such as low nuclear DNA content and low proportion of repetitive DNA (26-42%) (ShivaRaman and Rajekar, 1984). The 1C DNA content of Saccharum officinarum has been reported to be 4 g (Bennett and Smith, 1976). From the data on the repetitive DNA content in sugarcane and the data available in other Gramineae species, it is possible to comment on the relationship between the amount of repetitive DNA and nuclear DNA content in Gramineae. It can be seen from Fig.3.2 that the proportion of repetitive DNA varies linearly with DNA content. This is true for single copy DNA also. However, the slopes of two lines are different. Above a nuclear DNA content of 6 pg, the content of single copy DNA remains more or less constant while that of repetitive DNA continues to increase. Hence it appears that repeated DNA contributes significantly to the genome size in Gramineae.

Our data on DNA reassociation kinetics of the three sugarcane DNAs have revealed both similarities and specific differences in DNA during differentiation. The proportions of the slow reassociating DNA components and very rapidly reassociating DNA components are similar in all the three DNAs. Co-740 callus and CO-7219 callus DNAs, however, differ from CO-740 shoot DNA in the

FIGURE 3.2 RELATIONSHIP BETWEEN NUCLEAR DNA CONTENT
AND REPETITIVE DNA AND SINGLE COPY DNA
CONTENTS IN GRAMINEAE SPECIES

The data points used are from Table 3.2

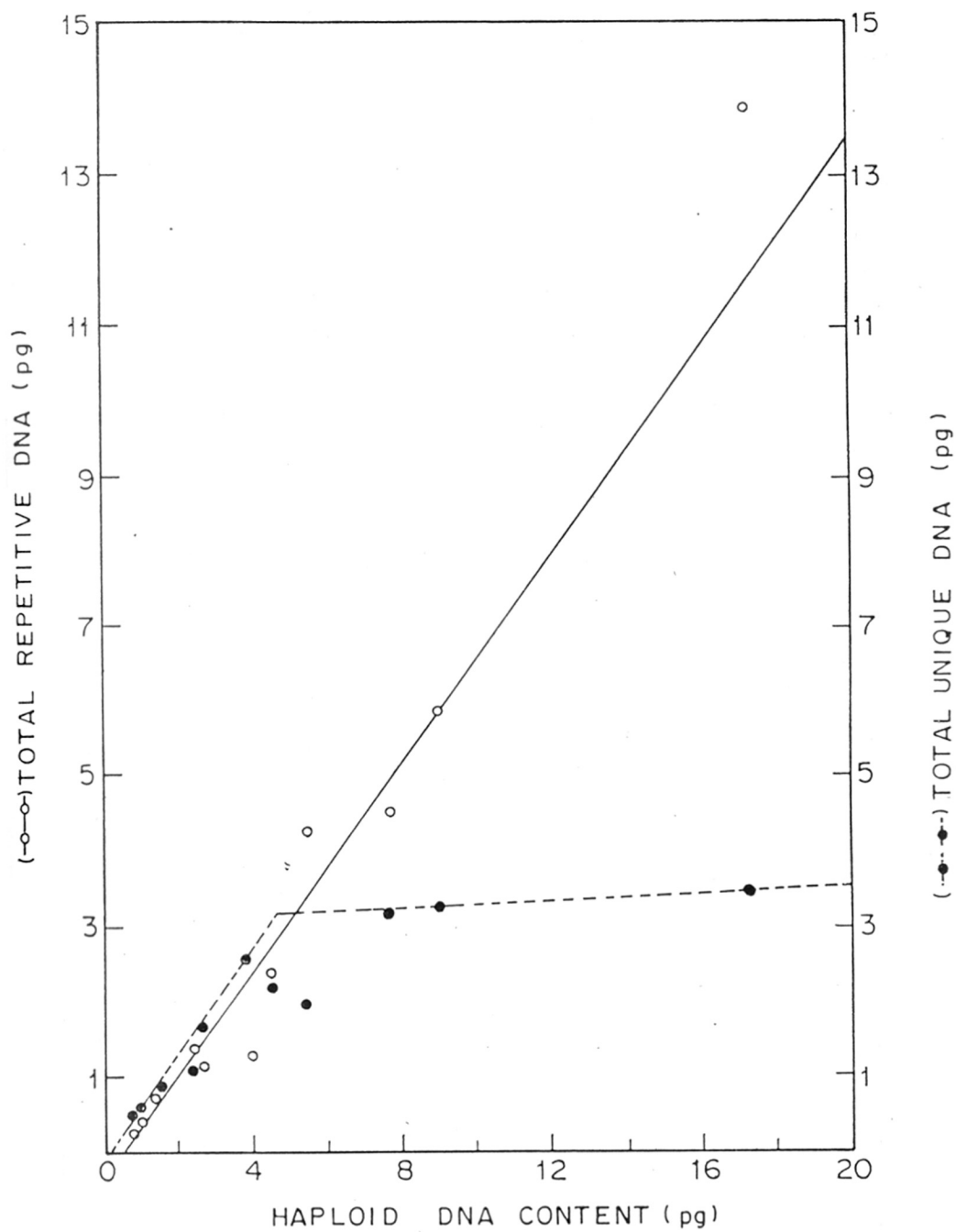


FIG. 3.2.

proportion of fast reassociating DNA component. The latter accounts for 16% CO-740 shoot and its frequency of reiteration and kinetic complexity are 58 and 5.70×10^4 nucleotide pairs respectively. The proportion of fast reassociating DNA is in the range of 38-47% of the total DNA for both the callus tissues. The kinetic complexity and the frequency of reiteration of this DNA are in the range of 8.99×10^5 to 2.28×10^6 nucleotide pairs and 209 to 726 respectively. Due to a significant change in the proportion of fast reassociating DNA, the proportion of total repetitive DNA in CO-740 shoot is 32% as against 62% in CO-740 callus. Since fast DNA includes mainly repeated DNA sequences, it can be concluded that repeated DNA sequences undergo a considerable change in their proportion and kinetic complexity during the transition from callus tissue to shoot tissue in sugarcane.

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CHAPTER IV

PHYSICO-CHEMICAL PROPERTIES OF REPETITIVE DNA
FROM CALLUS AND SHOOT TISSUES OF SUGARCANE

SUMMARY

Repeated DNA sequences were characterized in order to understand their properties and to assess the changes in them during in vitro differentiation in sugarcane. The thermal denaturation analysis of total repetitive DNA (Cot 100) of CO-740 callus, CO-740 shoot and CO-7219 callus showed the presence of one low and one high melting DNA component. Examination of thermal denaturation profiles of highly repetitive (Cot 0.1) and intermediately repetitive (Cot 0.1 - Cot 100) DNA fractions revealed that the high melting component was confined to Cot 0.1 fraction only.

The DNAs of different fragment length ranging from 0.5 kbp to 7.4 kbp were reassociated to Cot 100 and their melting patterns were studied. These data revealed that the melting temperature of repetitive duplexes varied in a narrow range of 85°C to 87°C for all the three sugarcane DNAs and that their hyperchromicity did not decrease much with the increase in DNA fragment length.

High resolution thermal denaturation analysis showed that repeated DNA sequences of the two callus tissues (CO-740 and CO-7219) were more heterogeneous than that of CO-740 shoot and that there was rearrangement in repeated DNA sequences during differentiation.

HPLC analysis of S1 nuclease resistant repetitive DNA revealed that the content of 5 mC was 2.7, 2.7 and 1.9 moles percent in CO-740 callus, CO-740 shoot and CO-7219 callus respectively. An additional peak was seen in CO-740 callus and CO-740 shoot DNAs, the amount being 1.1 moles percent and 4.2 moles percent respectively. This additional peak was not seen in CO-7219 callus DNA.

The mini Cot curve data of Cot 100 fraction indicated the presence of very fast and fast reassociating DNA components in CO-740 callus and shoot tissues and only fast reassociating DNA component in CO-7219 callus.

INTRODUCTION

In the previous two chapters, I have described the physicochemical characterization and kinetic complexity of DNAs from the three sugarcane tissues namely CO-740 callus, CO-740 shoot and CO-7219 callus. During these studies, all the three sugarcane DNAs exhibited similarities in their melting temperature as well as buoyant density. However, differences were noticed in the following three parameters: (i) The CO-740 shoot DNA differed slightly from the two callus DNAs in its behaviour towards AluI, Bam HI, EcoRI and Hind III. (ii) The proportion of 5 mC was less in CO-740 callus and shoot DNA than that in CO-7219 callus DNA. (iii) The content of fast DNA was less (16.5%) in CO-740 shoot DNA than that (38-47%) in the two callus DNAs.

In this chapter, I describe (i) the melting behaviour of different repeat DNA fractions to estimate sequence divergence among repeated DNA sequences (ii) the content of 5 mC in repetitive DNA using high performance liquid chromatography and (iii) mini Cot curves of total repetitive DNA fractions and those of highly repetitive fractions to identify the presence or absence of repeat families with discrete repetition frequency.

MATERIALS AND METHODS

Isolation of DNA and its shearing and sizing

DNAs were isolated from callus tissues of sugarcane cultivars CO-740 and CO-7219 as well as from shoots of sugarcane cultivar CO-740 by the procedure outlined in Chapter II. The molecular weight of native unsonicated DNA from the three sugarcane tissues was estimated to be approximately 10 kbp. However, trailing was often noticed in the range of 10 kbp to 0.5 kbp size. High molecular weight DNA was, therefore, obtained by direct elution method described in Chapter II.

Shearing of DNAs to an average size of 0.5 kbp and sizing of DNAs were carried out as detailed in Chapter III. DNA fragments in the range of 2.8 to 7.4 kbp were obtained using VirTis 60 K homogenizer by varying the conditions of speed and time between 30,000 to 58,000 RPM and 4 to 45 min respectively (Table 4.1). Sonication using the Biosonic III fitted with 6 mm probe for 1 min at 40 setting yielded DNA fragments of 1.5 kbp. The DNA concentrations were kept constant at 400-450 µg/ml. The average fragment size of sheared DNAs was determined by agarose slab gel electrophoresis in 1% agarose using HindIII digest of lambda DNA as molecular weight marker (Fig. 4.1)

TABLE 4.1 SONICATION OF SUGARCANE DNA TO OBTAIN DNA FRAGMENTS
OF DIFFERENT SIZES

Instrument used	Settting	Time (min)	Average DNA fragment size (kbp)
VirTis 60K homogenizer	32,000 RPM	4	7.4
"	30,000 RPM	30	5.2
"	48,000 RPM	7	4.6
"	50,000 RPM	15	4.2
"	57,000 RPM	45	2.8
Biosonic III fitted with 12 mm probe	70 setting	3	2.0
Biosonic III fitted with 6 mm probe	40 setting	1	1.5
Biosonic III fitted with 12 mm probe	maximum setting	3	0.5

The average DNA fragment size was determined by agarose gel electrophoresis. A few representative fragment sizes are shown in Fig. 4.1.

FIGURE 4.1 AGAROSE SLAB GEL ELECTROPHORESIS OF
SUGARCANE DNAs OF DIFFERENT FRAGMENT SIZES

- Lane 1. Unsheared native sugarcane DNA
- Lane 2. Average fragment size 5.2 kbp
- Lane 3. Average fragment size 4.2 kbp
- Lane 4. λ DNA digested with Hind III (23.1 kbp,
9.6 kbp, 6.6 kbp, 4.4 kbp, 2.3 kbp and
2.2 kbp)
- Lane 5 Average fragment size 2.8 kbp
- Lane 6 Average fragment size 1.5 kbp
- Lane 7 Average fragment size 0.5 kbp
- Lane 8 ϕ x 174 RF DNA digested with Hae III (1.36 kbp,
1.07 kbp, 0.872 kbp, 0.603 kbp and 0.310 kbp)

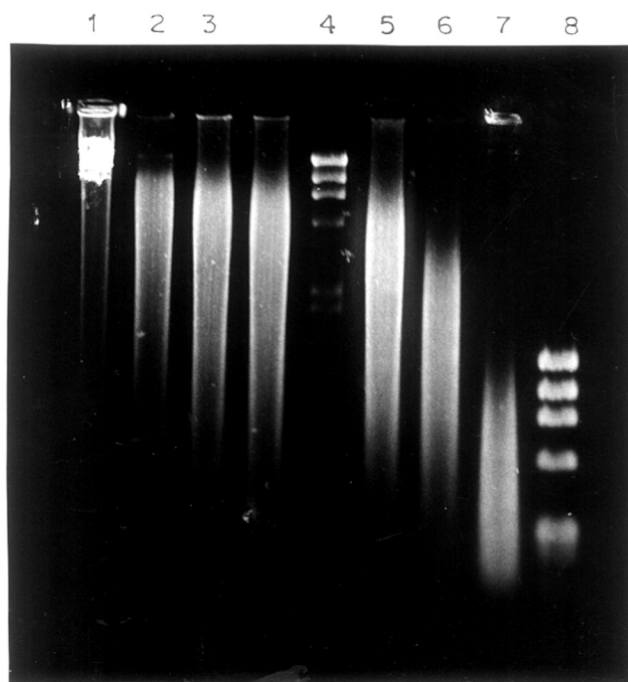


FIG. 4.1 .

Isolation of repetitive DNA fractions

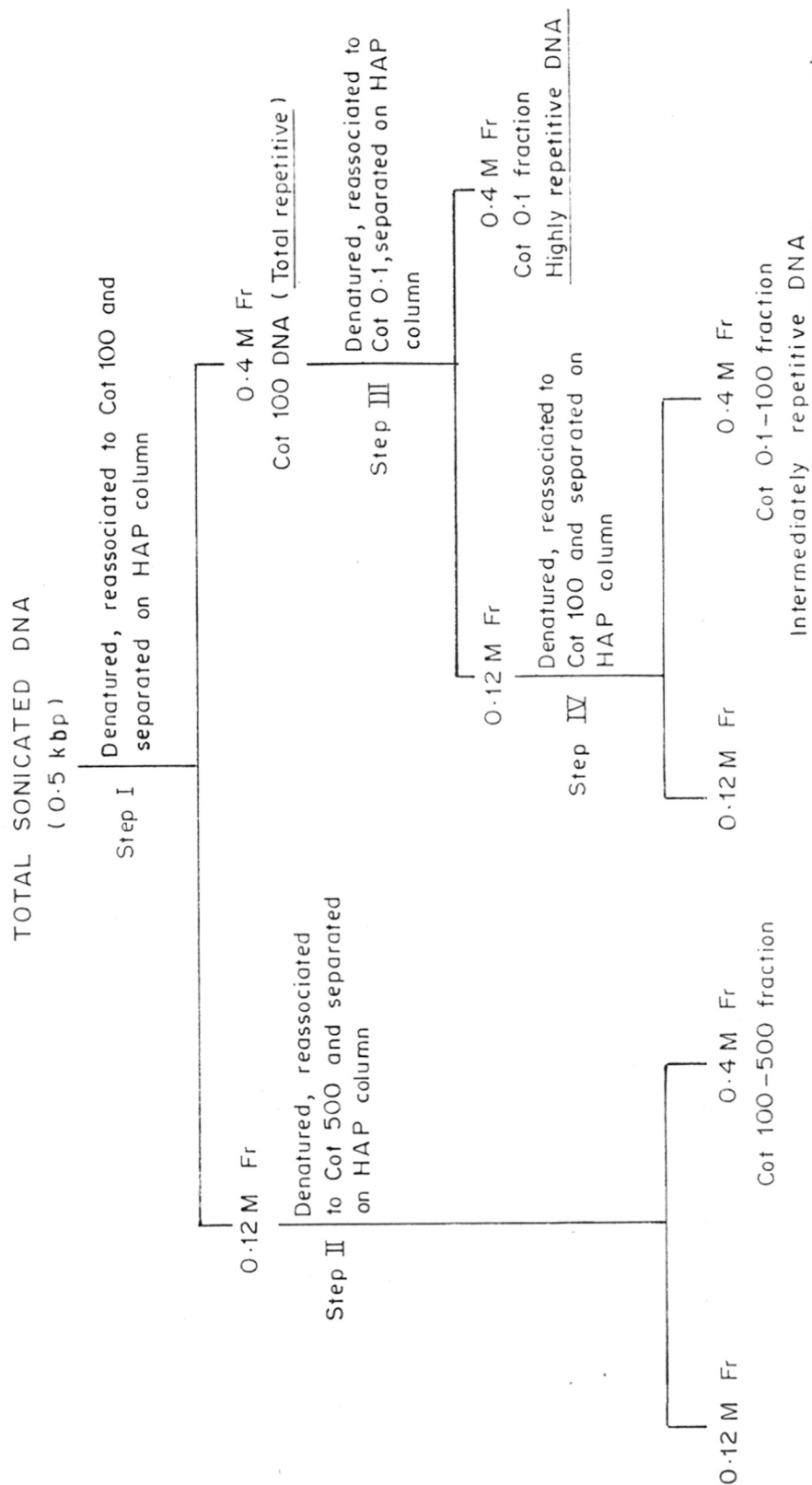
As shown earlier, the reassociation curves of 0.5 kbp long sugarcane DNAs from the three tissues could be modelled into one fast and one slow DNA component (Chapter III). Based on the comparison of $Cot \frac{1}{2}$ values, the fast fraction was shown to consist of mainly repeated DNA sequences. The very fast and fast DNA together represented the total repetitive DNA in sugarcane genome. This repetitive DNA was arbitrarily divided into highly repetitive (sequences reassociating by $Cot \ 0.1$) and intermediately repetitive (sequences reassociating between $Cot \ 0.1$ to 100) DNA fractions. The repetitive DNA in all the three sugarcane tissues thus includes DNA sequences reassociating by $Cot \ 100$. Single copy DNA sequences reassociating between $Cot \ 100$ - 500 were also obtained for a comparative study. All the two repetitive DNA fractions and single copy DNA were isolated by hydroxyapatite column chromatography as described in chapter III. The exact scheme of isolation of these fractions is shown in Fig. 4.2.

Thermal denaturation of native 0.5 kbp long DNAs and of repetitive DNA fractions

Thermal denaturation of various DNA samples was studied in 0.12 M sodium phosphate buffer (pH 6.8) at a heating rate of $1^{\circ}C/min$ using Gilford 250 spectrophotometer

FIG. 4.2.

FLOW DIAGRAM FOR FRACTIONATION OF SUGARCANE DNAS



as detailed in chapter II. Total hyperchromicity of DNA was normalised to 100% and the graph of percentage hyperchromicity versus temperature was plotted.

Calculations of T_m , hyperchromicity and base mismatch were done according to standard procedures (Zimmerman and Goldberg, 1977, Wimpee and Rawson, 1979, Britten et al. 1974).

For high resolution thermal denaturation analysis, DNAs were denatured at a heating rate $0.25^{\circ}\text{C}/\text{min}$ and absorbance values were recorded at 0.1°C increment, from 55°C to 100°C . The melting curves were obtained by plotting the temperature derivative of the absorbance of DNA against temperature (dA/dT vs T) as outlined in Chapter II. To check the reproducibility of the procedure, at least five DNA preparations each in duplicate were melted and the resulting curves were analyzed separately.

Isolation of S1 nuclease resistant repetitive DNA duplexes

These experiments were carried out according to the procedure of Murray et al. (1978) with some modifications. High molecular weight DNA (Fragment size 10 kbp) was obtained by direct elution method and was dialyzed against 0.18 M NaCl containing 0.006 M PIPES buffer (pH 6.8). The DNA samples were denatured in a boiling water bath for 10 min and then allowed to reassociate at

62°C to Cot 100. After reassociation, DNA samples were adjusted to 25 mM sodium acetate (pH 4.5), 0.1 mM ZnSO_4 and 25 mM 2-mercaptoethanol. (Goldberg *et al.* 1975). At least 1-1.5 mg of DNA was used for each experiment. After addition of S1 nuclease (0.1 u/ μg of DNA), the DNA samples were incubated for 30 min. at 37°C to digest single stranded DNA. The reaction was terminated by adjusting the samples to 0.12 M sodium phosphate buffer (pH 6.8) and simultaneously cooling to 4°C. S1 nuclease resistant DNA duplexes were then separated on HAP column by elution with 0.4 M sodium phosphate buffer (pH 6.8).

High performance liquid chromatography

Base composition analysis of S1 nuclease resistant repetitive DNAs was carried out using HPLC to determine the presence of 5-methyl cytosine and other odd bases, if any. DNA samples (100-200 μg) were hydrolyzed with 70% perchloric acid and HPLC runs were performed as described in Chapter II.

Mini Cot curves

The optical reassociation of repetitive DNA fractions, isolated at 62°C, was monitored in the Gilford 250 spectrophotometer as detailed in chapter III. The DNA samples were dialyzed extensively to 0.12 M sodium

phosphate buffer (pH 6.8) prior to optical reassociation studies. DNA concentrations were such that the absorbance after melting remained within the linear absorbance range of spectrophotometer. All optical reassociation data were evaluated using a computer aided non-linear regression procedure as described earlier in chapter III.

RESULTS

Thermal denaturation analysis of native sonicated DNA

Figure 4.3 depicts the melting curves of sonicated 0.5 kbp size sugarcane DNAs of all the three tissues. It is clear from this figure that the melting profiles of native 0.5 kbp DNAs are smooth and monophasic. The melting data obtained by analyzing such curves are summarized in Table 4.2. The native 0.5 kbp DNAs melt with an average melting temperature range of 84.5°C to 85.1°C and their hyperchromicities are in the range of 17.4 to 20.2%.

Melting analysis of repetitive DNA fractions

The thermal denaturation profiles of Cot 100 DNA fractions of the three sugarcane tissues are presented in Fig. 4.3. It can be seen from Fig. 4.3 that Cot 100 DNA fractions of the three sugarcane tissues exhibit biphasic melting profiles. The melting temperature of high melting fraction varies from 93.0°C to 93.2°C and its proportion is between 13-16% of the total genome. The melting temperature of low melting fraction is in the range of 75-77°C (Table 4.2). The average hyperchromicity of all the Cot 100 DNA fractions varies between 14-17%.

FIGURE 4.3 MELTING PROFILES OF NATIVE, SONICATED SUGARCANE DNA
AND COT 100 DNA

All melting experiments were carried out in
0.12 M sodium phosphate buffer (pH 6.8). The
temperature of the DNA was raised at a rate of
1°C/min.

Sonicated (0.5 kbp) DNA	0—0—0
Cot 100 DNA fraction	●—●—●
<u>E. coli</u> DNA	△—△—△

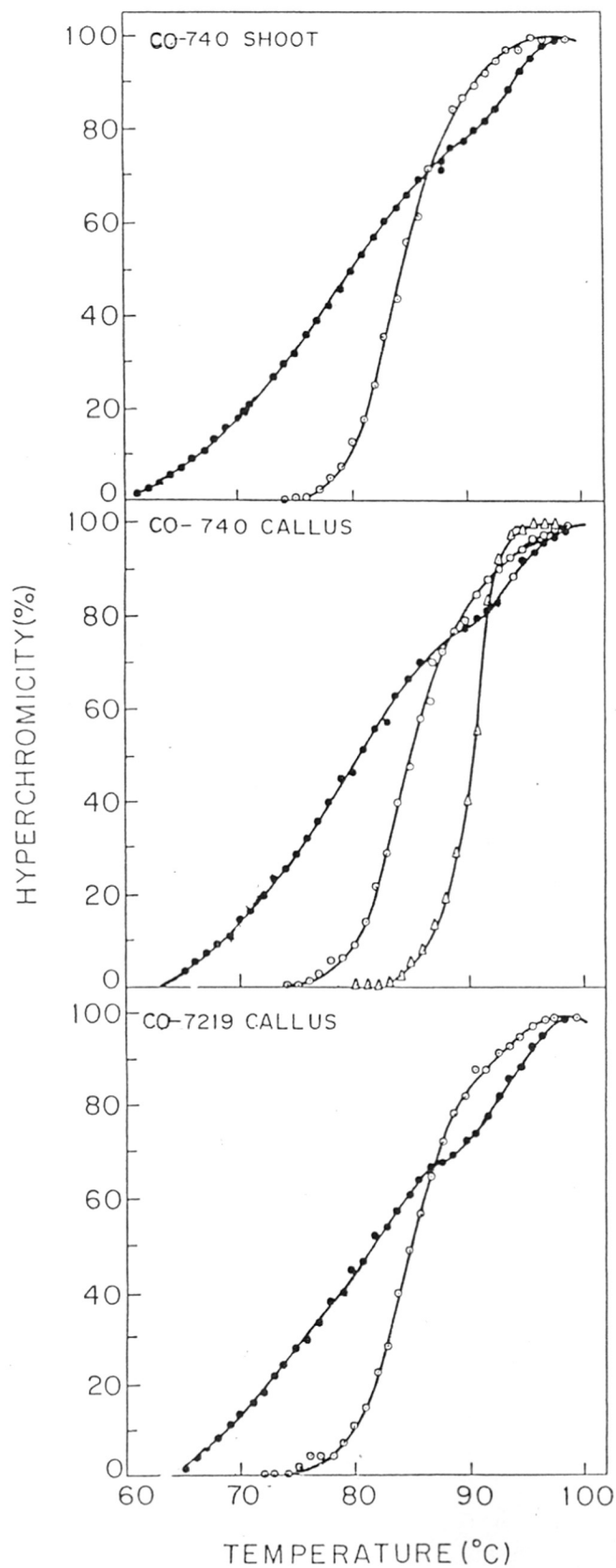


FIG. 4.3.

Figure 4.4 shows the thermal denaturation profiles of highly repetitive (Cot 0.1), intermediately repetitive (Cot 0.1 - 100) and single copy (Cot 100-500) DNA fractions. Here, the Cot 0.1 DNA exhibits a biphasic melting pattern with a minor high melting fraction (T_m 92°-96°C) and a major low melting fraction (T_m 77°-79°C). The melting profiles of Cot 0.1 - 100 DNA fractions of the three sugarcane DNAs, however, are monophasic with T_m of 74.9°-76.5°C. The single copy DNAs from the three sugarcane tissues reassociating between Cot 100-500 exhibit a monophasic melting curve with a melting temperature between 74.9°C and 77.4° (Figure 4.4). From these data, it is clear that the high melting fraction is mainly confined to the highly repetitive DNA fraction in all the three sugarcane tissues.

To estimate sequence divergence among repeated DNA sequences, the T_m of different repetitive DNA fractions was compared with that of the corresponding native sonicated DNA. As seen from Table 4.2, there is a lowering of T_m in all the repeat DNA fractions. The extent of lowering in T_m is, however, less (5.7°C - 7.5°C) in case of highly repetitive DNA than that in intermediately repetitive DNA (8.6°C - 9.6°C), total repetitive DNA (8.1°-8.8°C) and single copy DNA (7.1° - 9.6°C). From these data

FIGURE 4.4 MELTING PROFILES OF REPEAT AND SINGLE
COPY DNA FRACTIONS OF THREE SUGARCANE
TISSUES (IN 0.12 M SODIUM PHOSPHATE
BUFFER pH 6.8)

Cot 0.1 fraction (●—●—●)

Cot 0.1-100 fraction (0-0-0)

Cot 100-500 fraction (Δ-Δ-Δ)

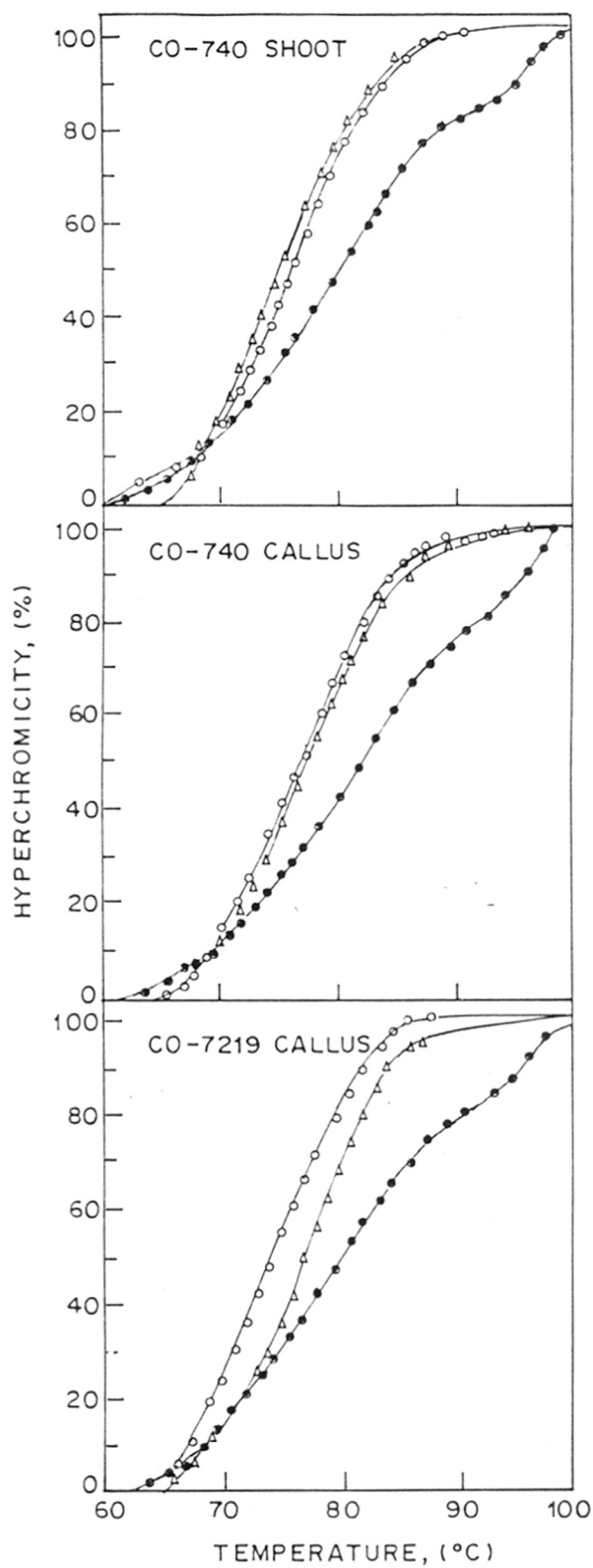


FIG. 4.4.

TABLE 4.2 THERMAL DENATURATION PROPERTIES OF NATIVE, SONICATED DNA AND REPETITIVE

DNA FRACTIONS

Type of DNA	Melting Temperature ^a (T _m °C)	Proportion of ^b the high melting DNA in the total genome (%)	Base mismatch ^c (%)
1	2	3	4
<u>CO-740 Callus</u>			
Native 0.5 kbp long DNA (5)	85.1 ± 0.3	-	-
Cot 100 (total repetitive) (7)	LM 77.0 ± 0.6 HM 93.2 ± 0.4	16.7 ± 4.0	8.1
Cot 0.1 (4)	LM 78.6 ± 0.2 HM 92.4 ± 1.8	- 7.7 ± 0.1	6.5 -
Cot 0.1 - 100 (4)	76.5 ± 0.9	-	8.6
Cot 100 - 500 (4)	76.9 ± 0.8	-	8.2
<u>Cot-740 Shoot</u>			
Native 0.5 kbp long DNA (8)	84.5 ± 0.5	-	-
Cot 100 (Total repetitive) (5)	LM 75.8 ± 0.5 HM 93.1 ± 0.1	- 12.8 ± 3.2	8.7 -
Cot 0.1 (4)	LM 78.8 ± 0.2 HM 96.0 ± 0.4	- 7.7 ± 0.3	5.7 -

TABLE 4.2 (continued)

1	2	3	4
Cot 0.1 - 100 (4)	75.4 ± 0.4	-	9.1
Cot 100 - 500 (4)	74.9 ± 0.7	-	9.6
CO-7219 Callus			
Native 0.5 kbp long DNA (6)	84.5 ± 0.5	-	-
Cot 100 (Total repetitive) (8)	LM 75.7 ± 0.7 HM 93.0 ± 0.1	- 15.3 ± 0.7	8.8 -
Cot 0.1 (4)	LM 77.0 ± 0.6 HM 93.9 ± 0.1	- 8.6 ± 0.5	7.5 -
Cot 0.1 - 100 (4)	74.9 ± 0.7	-	9.6
Cot 100 - 500 (4)	77.4 ± 0.2	-	7.1

Figures in parenthesis indicate the number of experiments performed

a - Obtained from Fig. 4.3 and 4.4

b - Obtained as $e = \frac{a \times d}{100}$

c - Calculated by using the relationship that 1°C lowering in T_m corresponds to 1% base mismatch. LM and HM stand for low melting and high melting fractions respectively.

it appears that the extent of sequence divergence is less among highly repeat DNA sequences than that in other DNA sequences.

Thermal denaturation of repetitive duplexes of varying fragment lengths

In order to study the effect of DNA fragment length on its melting temperature, thermal denaturation behaviour of sugarcane DNAs of different fragment length was studied. Figure 4.5 displays the melting profiles of the DNAs of different fragment lengths reassociated to Cot 100 Ms. and the results are summarized in Table 4.3.

Several interesting features emerge from these data. For example, the biphasic character of Cot 100 DNA obtained from the DNA fragment of 0.5 kbp size disappears with the increase in DNA fragment length. Furthermore, the T_m of the Cot 100 DNA fraction of fragment length ranging from 1.5 kbp to 7.4 kbp is comparable to that of native sonicated DNA. The hyperchromicity of these Cot 100 DNA fractions remains in the range of 15-20% and does not decrease with the increase in DNA fragment length. All these results imply the occurrence of very long stretches of repeated DNA sequences in sugarcane genome with very little base mismatch among them.

FIGURE 4.5 THERMAL DENATURATION PROFILES OF SUGARCANE DNAs OF DIFFERENT
FRAGMENT LENGTHS

The melting profiles of native 0.5 kbp long DNA (●—●) is included for comparison. Each point in these graphs represents an average of atleast three experiments. (X-X-X) represents duplexes isolated from 0.5 kbp long DNA (0-0-0) represents duplexes isolated from 4.6 kbp long DNA (A-A-A) represents duplexes isolated from 10 kbp long DNA

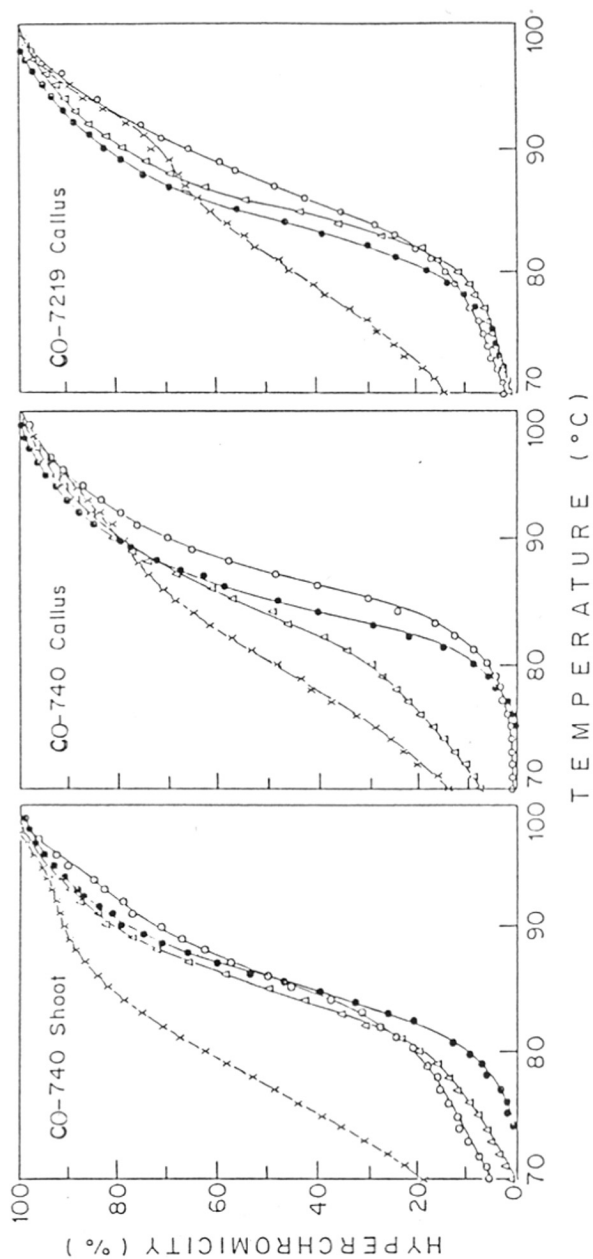


FIG. 4.5.

TABLE 4.3 MELTING ANALYSIS OF REPETITIVE DUPLEXES ISOLATED FROM DNAs OF
DIFFERENT FRAGMENT LENGTHS

Species and DNA fragment size (kbp)	Fraction ^a bound to HAP at Cot 100 M.s.	Hyperchromicity ^b	Tm ^c °C
1	2	3	4
<u>CO-740 callus</u>			
Native sonicated DNA (0.5 kbp)	-	20.2 ± 4.8	85.1 ± 0.3
0.5	0.58	20.0 ± 0.2	LM 78.0 ± 0.4 HM 93.6 ± 0.2
1.5	0.84	20.7 ± 0.3	86.9 ± 0.1
2.8	0.84	20.3 ± 0.4	87.0 ± 0.4
4.2	0.85	20.3 ± 0.7	87.1 ± 0.1
4.6	0.85	20.7 ± 0.3	87.2 ± 0.3
7.4	0.86	18.9 ± 1.3	87.3 ± 0.1
<u>CO-7219 callus</u>			
(0.5 kbp)	-	19.8 ± 4.7	84.5 ± 0.5
Native sonicated DNA	0.53	18.9 ± 1.1	LM 78.4 ± 0.1 HM 93.1 ± 0.7
0.5			

TABLE 4.3 (continued)

1	2	3	4
1.5	0.61	18.9 ± 1.3	LM 78.7 ± 1.9 HM 94.0 ± 0.6
2.8	0.69	18.9 ± 0.7	86.6 ± 0.5
4.2	0.72	18.2 ± 0.9	86.2 ± 0.4
4.6	0.73	18.5 ± 0.4	87.2 ± 0.1
5.2	0.75	18.7 ± 0.7	86.8 ± 0.4
7.4	0.80	16.9 ± 2.0	86.6 ± 0.1
<u>CO-740 shoots</u>			
Native sonicated DNA (0.5 kbp)	-	17.4 ± 5.6	84.5 ± 0.5
0.5	0.61	15.1 ± 0.3	LM 75.8 ± 0.5 HM 93.1 ± 0.1
2.0	0.77	17.6 ± 0.2	85.1 ± 0.7
2.8	0.73	16.6 ± 0.7	85.7 ± 0.1
4.2	0.81	16.4 ± 0.8	85.1 ± 0.2

TABLE 4.3 (continued)

1	2	3	4
4.6	0.83	16.7 $\pm$ 1.5	86.0 $\pm$ 0.1
5.2	0.77	16.2 $\pm$ 1.1	85.5 $\pm$ 0.9

a - Obtained from Fig. 5.3

b - Obtained using the formula as described in the caption in Table 2.1, Chapter II.

c - Obtained from Figure 4.5 of their respective denaturation profiles.

High resolution thermal denaturation of repetitive DNAs

To obtain more information about thermal stability and heterogeneity among repeated DNA sequences, Cot 100 fractions of the three sugarcane DNAs were extensively dialysed in 0.12 M sodium phosphate buffer (pH 6.8) and thermally denatured at a rate of 0.25°C/min as described earlier in chapter III with the exception that the heating was started from 55°C onwards. The melting curves thus obtained were differentiated using the first derivative analysis.

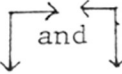
Figure 4.6 depicts the derivative melting profiles of repetitive DNA (Cot 100 DNA) fractions. These profiles are broad and reveal the presence of thirty four to forty seven components which were divided into two groups; the first group includes thermal components upto 86.4°C (the T_m of native unsonicated DNA) and the second group includes thermal components from 86.4°C to 97°C. There are thirty to thirty five components in the first group and twelve to fourteen components in the second group in the two callus cultures. In case of shoot DNA, twenty four and ten components are present in the first and second group respectively indicating thereby that the shoot DNA is less heterogeneous as compared to two callus DNAs.

A qualitative comparison of the derivative melting profiles of Cot 100 DNA was next made by plotting a graph

FIGURE 4.6 THE HRTD PROFILES OF SUGARCANE REPETITIVE DNA

The HRTD profiles of Cot 100 DNA fractions were obtained with a heating rate of $0.25^{\circ}\text{C}/\text{min}$ and the absorbance was recorded continuously at 0.1°C temperature intervals. The temperature derivative of absorbance was determined as outlined in Chapter II.

The dashed line (-----) indicates the DNA melting curve at a heating rate of $1^{\circ}\text{C}/\text{min}$.

 Indicate the direction of the scale that was used.

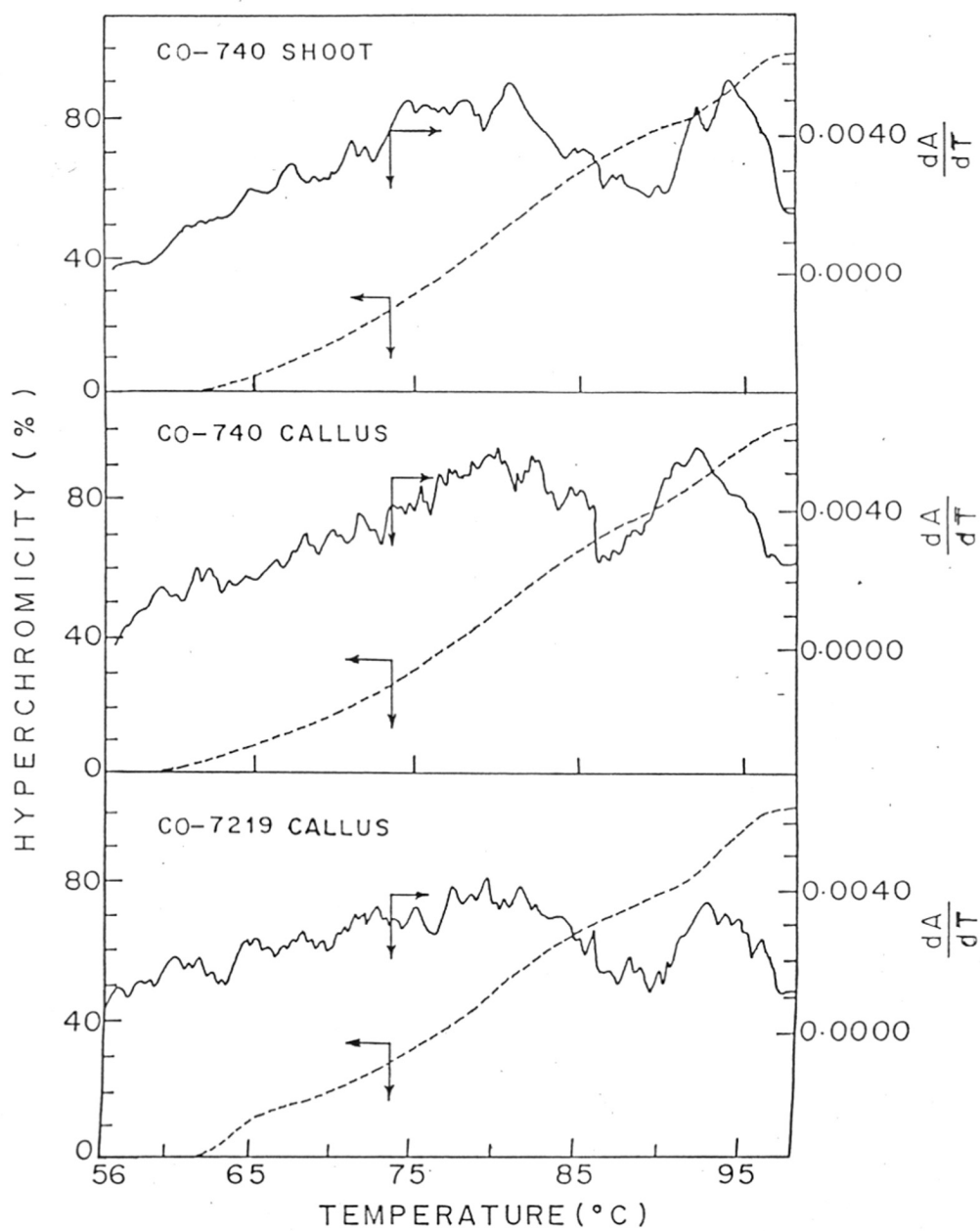


FIG. 4.6.

indicating the positions of peaks and determining the number of peaks sheared among the three sugarcane tissues (Fig. 4.7). A temperature span of 0.2°C was considered to estimate the number of components that are shared and the data are summarized in Table 4.4. The DNA components melting before 86.4°C (T_m of native sonicated DNA) are considered to be A+T rich while those melting after 86.4°C are G+C rich. As seen from Table 4.4, thirteen components in A+T rich region and three components in G+C rich region are common in all the three sugarcane DNAs. Twenty four components in A+T region and eight components in G+C region are commonly shared by CO-740 callus and CO-7219 callus cultures. In CO-740 callus and CO-740 shoot cultures, twenty components in A+T region and five in G+C region are common. These data thus clearly indicate that thermal components having identical positions among all the three sugarcane tissues are more on A+T rich side than that on G+C rich side.

Analysis of mini Cot curves

In order to get more information about the number of components present in Cot 100 DNA fraction, its optical reassociation was studied along with native sonicated DNA. The reassociation kinetics data of Cot 100 DNA fractions depicted in Fig. 4.8 and summarized in Table 4.5 indicate the presence of very fast and fast reassociating

FIGURE 4.7 THE POSITIONS OF MELTING COMPONENTS OBSERVED IN THE HRTD
PROFILES OF SUGARCANE REPETITIVE DNAs

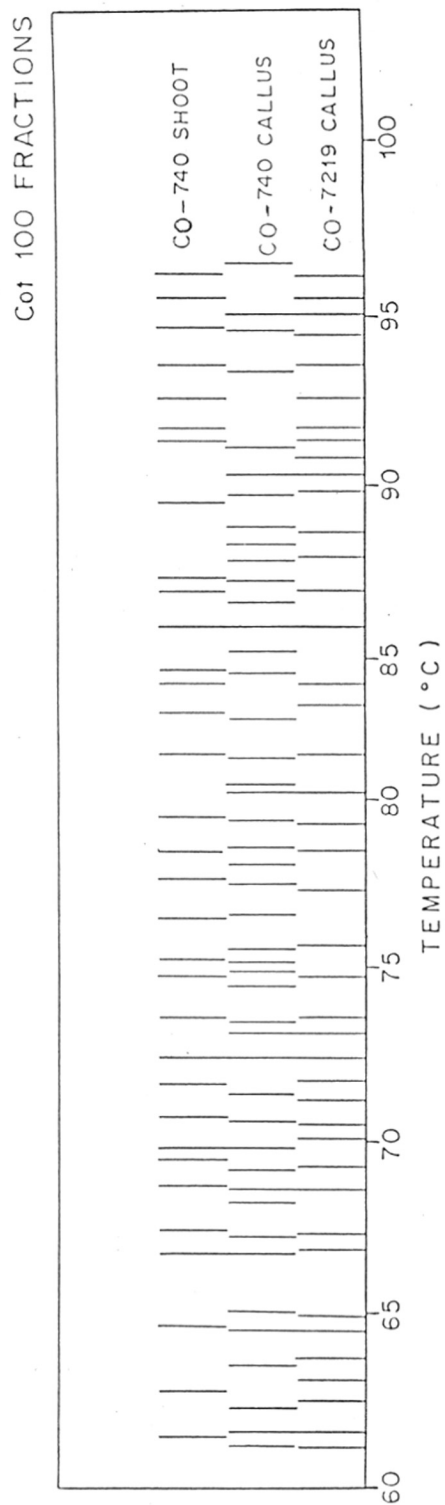


FIG. 4.7.

TABLE 4.4 DISTRIBUTION OF THE COMPONENTS IN THE FINE MELTING
PROFILES OF COT 100 DNA FRACTION ON EITHER SIDE OF
86.4°C

DNA	Number of components ^a		Total
	A+T rich side	G+C rich side	
CO-740 callus	35	12	47
CO-740 shoots	24	10	34
CO-7219 callus	30	14	44
Components shared by 3 sugarcane tissues	13	3	16
Components shared by CO-740 callus and shoot	20	5	25
Components shared by CO-740 callus and CO-7219 callus	24	8	32

a - obtained from Fig. 4.7

DNA components in CO-740 callus and CO-740 shoot and only fast reassociating DNA component in CO-7219 callus. The frequency of reiteration and the kinetic complexity of very fast reassociating DNA components are 2.4×10^5 and 1.0×10^3 np respectively in CO-740 callus while CO-740 shoot has a frequency of reiteration of 7.5×10^3 and kinetic complexity as 4.6×10^2 np. The fast reassociating DNA component present in all the three systems has a kinetic complexity ($1.1 - 2.9 \times 10^5$ np) and frequency of repetition ($4.1 \times 10^1 - 4.8 \times 10^3$). The isolated repeat DNA fractions from all the three sugarcane tissues show an increase in the rate of reassociation and copy number (Table 4.5) as against the values obtained for the same components from the reassociation kinetics curves of total sonicated sugarcane DNA (Table 3.1, Chapter III). This is mainly because kinetics of repetitive DNA fractions are affected to some extent when studied in the presence of other repetitive fractions of varying frequency or in the presence of single copy DNA.

HPLC of S1 nuclease resistant repeat DNA sequences

After determining the thermal heterogeneity and kinetic complexity of repeated DNA sequences, the latter were next characterized with respect to the content of 5-methyl cytosine. For this purpose, S1 nuclease resistant

FIGURE 4.8 REASSOCIATION KINETICS CURVES OF SUGARCANE
REPETITIVE DNAs

Optical reassociation of Cot 100 fraction of CO-740 callus, CO-740 shoot and CO-7219 callus was performed in 0.12 M sodium phosphate buffer (pH 6.8). Percent reassociation was calculated from the drop in optical density and Cot values were derived from the time of incubation and DNA concentration. The solid line through the data points represents the best least squares fit, allowing all the parameters to free float. The lower dashed curves represent the predicted reassociation kinetics of pure components.

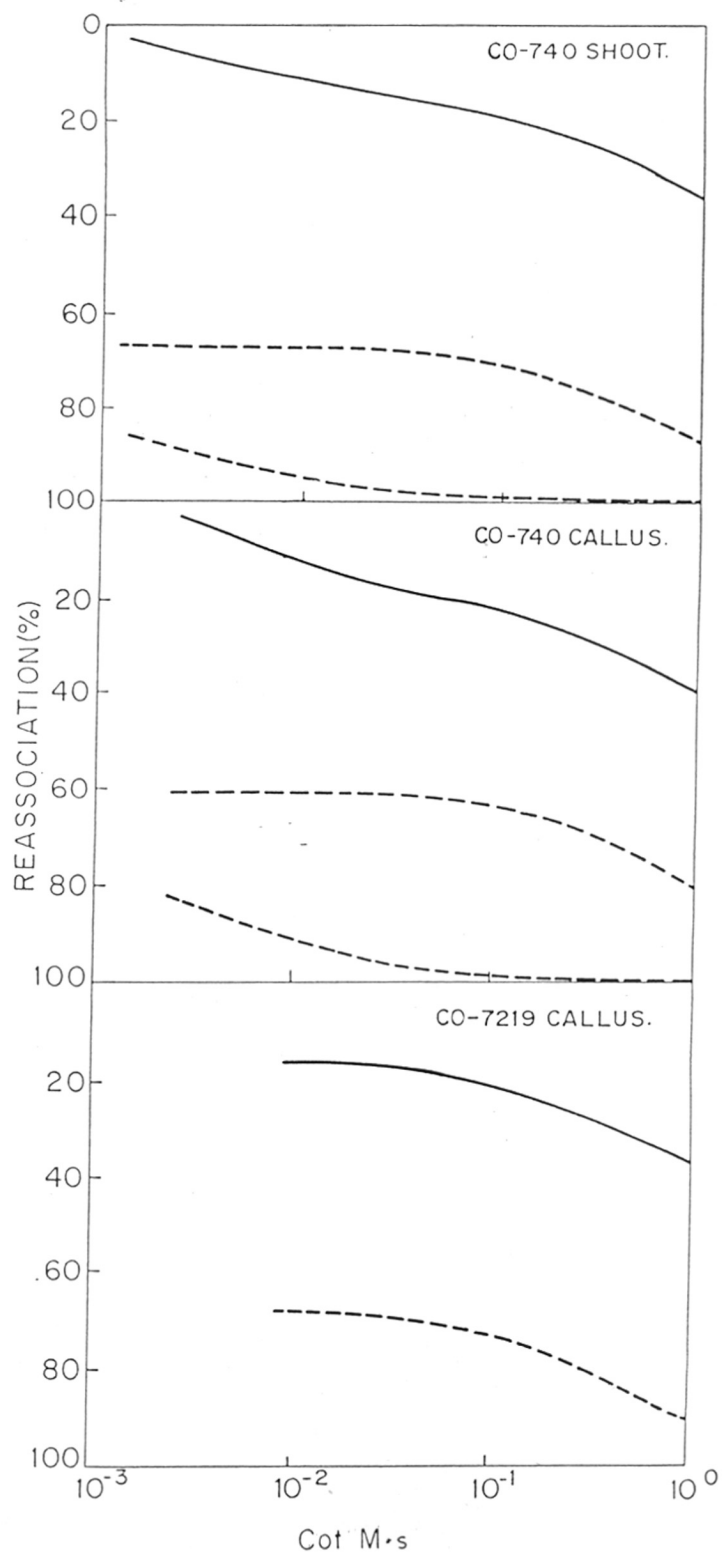


FIG. 4.8.

TABLE 4.5 KINETIC ANALYSIS OF MINI COT CURVES OF COT 100 SUGARCANE DNAs

Cot 100 DNA fraction	Fraction of DNA ^a	Cot $\frac{1}{2}$ observed ^a M _s	Cot $\frac{1}{2}$ pure ^b M _s	Frequency of repetition ^c	Kinetic complexity ^d nucleotide pairs
1	2	3	4	5	6
<u>CO-740 callus</u>	-	-	-	-	-
Very fast reassociating fraction (Cot 2.6 x 10 ⁻³ to 2.6 x 10 ⁻¹ M.s)	0.25	5.45 x 10 ⁻³	1.37 x 10 ⁻³	2.45 x 10 ⁵	1.02 x 10 ³
Fast reassociating fraction (Cot 2.6 x 10 ⁻¹ to 1 x 10 ⁰ M.s)	0.39	9.90 x 10 ⁻¹	3.90 x 10 ⁻¹	1.35 x 10 ³	2.92 x 10 ⁵
Unreassociated, (Cot > 1.0 M.s.)	0.31	-	-	-	-
<u>CO-740 shoot</u>					
Very fast reassociating fraction (Cot 3 x 10 ⁻³ to 1 x 10 ⁻¹ M.s)	0.17	3.62 x 10 ⁻³	6.24 x 10 ⁻⁴	7.54 x 10 ³	4.68 x 10 ²
Fast reassociating fraction (Cot 1 x 10 ⁻¹ to 1.0 x 10 ⁰ M.s)	0.33	6.57 x 10 ⁻¹	2.18 x 10 ⁻¹	4.13 x 10 ¹	1.63 x 10 ⁵
Unreassociated fraction (Cot > 1.0 M.s.)	0.46	-	-	-	-

TABLE 4.5 (continued)

1	2	3	4	5	6
<u>C0-7219 callus</u>	-	-	-	-	-
Fast reassociating fraction (Cot 9×10^{-3} to 1.0×10^0 M.s.)	0.32	4.64×10^{-1}	1.48×10^{-1}	4.80×10^3	1.10×10^5
Unreassociated (Cot $> 1.0 \times 10^0$ M.s.)	0.51	-	-	-	-

a - Values obtained from Fig. 4.8

b - Cot $\frac{1}{2}$ values observed x fraction of the genome

c-Cot $\frac{1}{2}$ of unique DNA (obtained from Fig. 3.1 of Chapter III) divided by Cot $\frac{1}{2}$ values of the given number fraction

d - Values obtained from the standard relation for E. coli where the Cot $\frac{1}{2}$ is 4.6 M.s.
(under our experimental conditions) and genome size is 4.5×10^6 nucleotide pairs

repetitive DNA duplexes were used.

Figure 4.9 shows the HPLC profiles of S1 nuclease resistant repeat DNA sequences. The amount of 5-methyl cytosine calculated from the peak area indicates that CO-740 callus, CO-740 shoot and CO-7219 callus contain 2.7, 2.7 and 1.9 moles percent of 5-methyl cytosine respectively (Table 4.6). Besides A,T,C,G and 5mC peaks, one additional peak is seen with CO-740 callus and CO-740 shoot DNA hydrolysate. This peak represents the modification of a base, its amount being 1.1 moles percent in CO-740 callus and 4.2 moles percent in CO-740 shoot DNA. Such an additional peak is not seen in CO-7219 callus DNA hydrolysate. In the absence of authentic samples of different possible modified bases, the identity of this peak or base could not be confirmed. That this peak may be an artifact of DNA isolation conditions or the chromatographic conditions was ruled out by the observation that this peak was not seen in the profile of calf thymus DNA (Fig. 2.8, Chapter II) which was hydrolysed and chromatographed under identical experimental conditions.

FIGURE 4.9 HPLC (REVERSED PHASE) SEPARATION OF BASES FROM
THE HYDROLYSATES OF S1 NUCLEASE RESISTANT
COT 100 DNA FRACTIONS OF SUGARCANE

A - ADENINE

C - Cytosine

G - Guanine

T - Thymine

↓ - Indicates 5 mC peaks

↓ - Indicate odd peaks

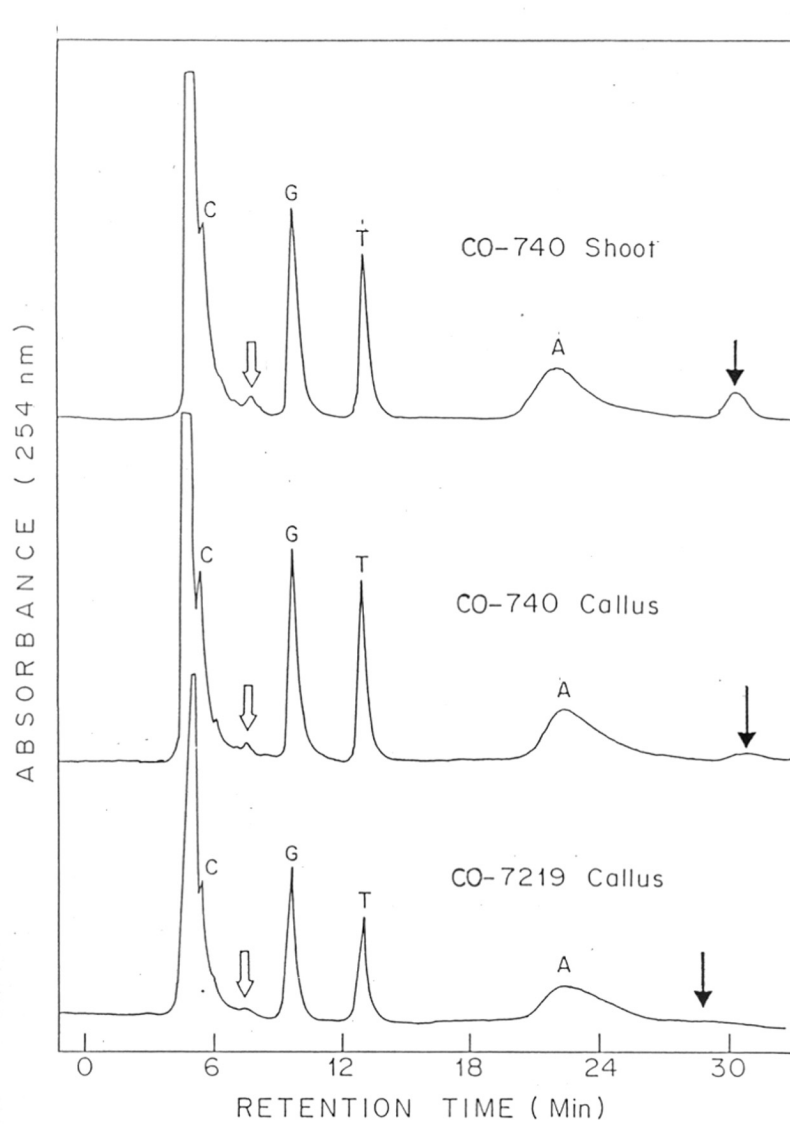


FIG. 4.9.

TABLE 4.6 CONTENT OF 5 mC IN S1 NUCLEASE RESISTANT REPETITIVE DNAs OF
SUGARCANE TISSUES

	Cot 100 DNAs		
	CO-740 callus	CO-740 shoot	CO-7219 callus
% 5 mC	2.73	2.71	1.91
% odd base	1.11	4.26	-

Proportions of 5-mC and odd base were calculated from the area under the
respective peaks in Fig. 4.9

DISCUSSION

In the present study, I have made an attempt to assess the differences at repetitive DNA level in the three sugarcane tissues namely CO-740 callus, CO-740 shoot and CO-7219 callus using approaches like thermal denaturation, fine melting, mini Cot curve and high performance liquid chromatography. Earlier, thermal denaturation and mini Cot curve analyses have been used in case of repetitive DNA isolated from millets (SivaRaman and Ranjekar, 1984, SivaRaman et al. 1984, Gupta and Ranjekar, 1981, 1982). Too scanty data are, however, available on the changes in repeated sequences during differentiation and dedifferentiation processes. Zheng et al. (1987) have recently studied the modulation of highly repeated sequences in rice when passing from in vivo (leaves) to in vitro (suspension cultured cells) stage. To my knowledge, ours is the first effort to use the high performance liquid chromatography and high resolution thermal denaturation techniques to examine the problem of differentiation by using repeat DNA sequences of the three sugarcane tissues. Some of the important findings are discussed in the following paragraphs.

Base mismatch and sequence divergence in sugarcane

Thermal denaturation studies have indicated that the total repetitive DNA fractions in sugarcane exhibit

a base mismatch of 8-8.7%. It is generally believed that the degree of base mismatch in a given DNA fraction gives an indication of the extent of sequence divergence. In family Gramineae, thermal stability of repetitive duplexes has been reported in a number of species such as wheat, oat, rye, barely, maize, finger millet, pearl millet, barnyard millet, fox tail millet and little millet (Deshpande and Ranjekar, 1980; Ranjekar et al., 1974, 1976; Smith and Flavell, 1975, Hake and Walbot, 1980; SivaRaman et al., 1984). Among these species, wheat, oat, rye, barley and maize show as much as 12-15% base mismatch in their repetitive duplexes indicating the occurrence of an extensive sequence divergence. In sugarcane, repeated sequences exhibit a moderate sequence divergence of about 8-9%. A similar degree of sequence divergence has been reported in maize (Hake and Walbot, 1980) and millets (SivaRaman and Ranjekar, 1984). It is known that sequence divergence accumulates with time and gives an idea about the age of a repeat family (Thompson and Murray 1980). From the data in sugarcane, it appears that repeat sequences in sugarcane are more of recent origin than those in other grasses mentioned above.

Thermal stability of repetitive duplexes of varying fragment lengths

From the thermal stability data of repetitive duplexes of varying fragment lengths, it is clear that the melting temperatures of repeated DNA sequences isolated from the DNAs of fragment length greater than 1.5 kbp are higher than that of native DNAs. An attempt was made to find out if this increase in T_m was due to increase in DNA fragment length. The melting temperatures of the repetitive duplexes can be predicted using the formula $T_m - T_n = \frac{B}{L}$ where T_n is the melting temperature of native DNA, L is the fragment length and B is a constant equal to 650 (Britten et al. 1974). Using this formula, the melting temperatures should be in the range of 83-85°C for the three sugarcane tissues. This range is much narrower than the observed range of 75-87°C. A likely explanation for this observation is that much of the repeated duplexes of these sugarcane tissues are present in long arrays consisting of smaller elements varying slightly in their nucleotide sequences. When the DNAs are sheared, there is a greater probability of a mismatch in the reassociated duplexes than if the entire stretches were left intact. This is actually the case in sugarcane as is evident from the previous data on thermal stability of Cot 100 fractions isolated from

sonicated sugarcane DNA. The high thermal stability of long repetitive duplexes is probably due to the fact that they are just zipped together to form stable duplexes leading to a minimum base mismatch among the shorter elements.

Occurrence of a high melting DNA component

Analysis of the individual Cot fractions which include DNA sequences reassociating in a narrow Cot range has enabled us to pin point specific differences which otherwise cannot be detected. Native sonicated 0.5 kbp DNA of the three sugarcane tissues has a smooth and monophasic melting profile. However, the repetitive Cot fractions show a biphasic melting profile in all the three DNAs. The minor high melting component (T_m 92.4° to 96°C) constitutes 12.9-16.7% of the total genome of these tissues and is present mainly in the highly repetitive DNA (Cot 0.1) fraction.

The presence of high melting DNA fraction has been shown in millets (Deshpande and Ranjekar, 1980; SivaRaman and Ranjekar, 1984, 1986). Millets are known to grow under arid conditions and can survive on poor and thin soil. The high melting component may, therefore, be playing an important role in the ecological adaptations of millets. In sugarcane, however, the situation is

quite different. Sugarcane requires high temperatures, plenty of water, sun light and high fertility soil. We, therefore, cannot explain the probable role of high melting fraction in sugarcane genome.

Fine melting analysis of repetitive DNA

Due to a very high sensitivity of HRTD technique, this approach has a great potential for the detection of small amount of differentially replicating DNA which is known to occur in a few plant species during in vitro differentiation. Though fine melting profiles of the three sugarcane DNAs are polyphasic, distinct differences and similarities can be observed in them. The total number of thermal components, for example, decrease from 47 in CO-740 callus to 34 in CO-740 shoot indicating a merger of the individual components as a result of differentiation of callus to shoot. In order to define which portion of the genome undergoes merging, Fig. 4.7 was constructed which shows the exact position of the melting components in HRTD profiles. The total number of components in A+T rich and G+C rich regions of sugarcane DNAs are tabulated in Table 4.4. It can be seen from Table 4.4 and Fig. 4.7 that there is a significant decrease in A+T rich components during differentiation thus indicating that the A+T rich region is affected during merging of components. Our

results also indicate that repeat DNA isolated from the two callus tissues is more heterogeneous than that of CO-740 shoot.

To our knowledge, it is for the first time that HRTD approach is used to assess the changes in DNA during in vitro differentiation in plants.

Absence of a distinct class of repetitive DNA in sugarcane CO-7219 callus

An examination of the mini Cot curves of Cot 100 DNA fraction from all the three sugarcane tissues reveals the absence of a very fast component in CO-7219 callus tissue. The HPLC profiles have also indicated the absence of any odd bases in this tissue. The very fast DNA component and the odd bases are present in the other two tissues namely CO-740 callus and CO-740 shoot.

As already explained, CO-7219 callus loses its capacity for differentiation within one year of subculture while CO-740 callus retains its capacity for differentiation even after 10 years of subculture. A question, therefore, can be asked whether the absence of very fast component and odd bases have any role in the differentiation phenomenon? Further work in this direction is being undertaken in our laboratory.

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CHAPTER V

DNA SEQUENCE ORGANIZATION IN CALLUS AND SHOOT TISSUES OF SUGARCANE

SUMMARY

DNA sequence organization patterns were studied in three sugarcane tissues, namely CO-740 callus, CO-740 shoot and CO-7219 callus. The reassociation kinetics of 10 kbp long DNA fragments showed that 75-86% of the sugarcane genomes consisted of alternating single copy and repetitive DNA sequences. The length of interspersed single copy DNA sequences varied in a narrow range of 1.4-2.2 kbp. The size distribution of S1 nuclease resistant repetitive duplexes isolated from long unsheared DNA fragments revealed two major size classes of repeats: (>1.5 kbp and 0.7 kbp to 1.1 kbp). A third size class (<0.2 kbp) was also present though in small amounts (6-20%).

The genome size (1C) of sugarcane is 4 pg and the DNA sequence organization is of mixed type. These data substantiate the trend in plants, namely, plants with 1C nuclear DNA content of less than 5 picograms have diverse patterns of sequence organization while those with 1C nuclear DNA content greater than 5 picograms have predominantly a short period interspersion pattern.

INTRODUCTION

After knowing that eukaryotic genomes consist of repeated and non-repeated DNA sequences, it is of interest to know how these sequences are arranged or distributed with respect to each other and what are their approximate lengths. DNA sequence organization refers to such information and this is expected to throw light on several important questions including gene regulation, genome evolution and cell functions in general.

The occurrence of interspersion of repetitive and single copy DNA sequences was first demonstrated by Britten and Kohne (1968). Later studies revealed the occurrence of two main types of interspersion patterns. Short period pattern or Xenopus pattern (Davidson et al., 1973) is characterized by the presence of repeat units of 0.2 to 0.4 kbp interspersed with single copy DNA sequences of 1-2 kbp. In long period or Drosophila pattern (Manning et al., 1975), repetitive DNA is clustered into 4-10 kbp units that are flanked by 10 kbp long single copy DNA sequences. The occurrence of short period interspersion pattern was initially demonstrated in Xenopus (Davidson et al., 1973), sea urchin (Graham et al., 1974), house fly (Efstratiadis et al., 1976), fishes (Olmo et al., 1982), mammals (Mayfield et al., 1980, Avvedimento et al., 1976; Schmid and Deininger, 1975) and Dictyostelium (Firtel and Kindle, 1975). Subsequent studies, however, showed the

the occurrence of long period interspersed pattern in a wide variety of unrelated organisms such as birds (Epplen et al., 1978; Wagenman et al., 1981), nematodes (Schachat et al., 1978) and fungi (Ullrich et al., 1980; Harshey et al., 1979; Hudspeth et al., 1977; Krimlauf and Marzluf, 1979).

Plants in general exhibit a great diversity in their sequence organization patterns. Maize (Hake and Walbot, 1980), wheat (Flavell and Smith, 1976) and rye (Smith and Flavell, 1977), for example, exhibit predominantly a short period interspersed pattern while many other plants have either mixed or long period interspersed patterns. The genomes of rice (Gupta et al., 1981, Dhar et al., 1988), flax (Cullis, 1981) and Cucumber (Lagu, 1987) are unique because they do not exhibit interspersed of repeated and single copy DNA sequences at a DNA fragment size of 20, 10 and 5 kbp respectively.

I undertook the DNA sequence organization studies in sugarcane system for the following two reasons. First, I wished to determine the arrangement of repeated and single copy DNA sequences in sugarcane and then compare it with those in other well characterized Gramineae species, namely wheat, maize rye and millets (SivRaman and Ranjekar, 1984, 1986). Secondly, I wanted to assess the changes, if any, that might have occurred in the sugarcane genome during

differentiation from callus tissue to shoot tissue. For this purpose, reassociation kinetics of long DNA fragments of CO-740 callus, CO-740 shoot and CO-7219 callus tissues were compared to those of short size (0.5 kbp) DNA fragments. Another set of experiments was performed in which S1 nuclease resistant DNA duplexes were isolated and sized by Agarose A-50 column chromatography. Finally the length of interspersed single copy DNA sequences was determined by studying reassociation of DNAs of different fragment length at Cot 100 M.s.

MATERIALS AND METHODS

DNA Isolation

DNAs from the three sugarcane tissues were obtained according to the procedure outlined in Chapter II.

Reassociation kinetics of long DNA fragments

The reassociation kinetics of long DNA (10 kbp) fragments were determined using hydroxyapatite column chromatography in the Cot range of 1×10^{-1} to 1×10^2 M.s. The experimental points were fitted by non-linear least squares regression analysis as described in Chapter III.

Isolation and sizing of S1 nuclease resistant repetitive DNA duplexes

These experiments were carried out according to the procedure of Murray et al. (1978) with some modifications as described in Chapter III. The size distribution of S1 nuclease resistant Cot 100 DNA duplexes was determined by gel filtration on Agarose Biogel A-50 (100-200 mesh size) column previously calibrated with calf thymus DNA of known duplex lengths.

During the preparation of Agarose column, the Agarose gel matrix (fully hydrated in 0.001 M Tris EDTA buffer) was poured around 5 mm glass beads in a 92 cm x 1.5 cm column. The column was equilibrated with 0.12 M sodium, phosphate buffer (pH 6.8) and was reused a number of times

after thorough washing.

Approximately 70-100 μg of S1 nuclease resistant repetitive DNA duplexes, separated by hydroxyapatite column chromatography, were loaded on the Agarose column and elution was carried out with 0.12 M sodium phosphate buffer (pH 6.8). The effluent was continuously monitored with the help of LKB 2138 UVICORD at 253 nm (Sachs and Painter, 1972). The exclusion limit of Agarose A-50 was found to be 1.5 kbp.

RESULTS

Reassociation kinetics of long DNA fragments

The presence or absence of interspersed repetitive and single copy DNA sequences can be determined by comparing the hydroxyapatite binding data of long and short DNA fragments over a range of Cot values. An increase in binding to hydroxyapatite as a function of DNA fragment length is considered to be an indication of the occurrence of interspersed repetitive and single copy DNA sequences (Davidson and Britten, 1973; Wetmure and Davidson, 1968).

Figure 5.1 shows the reassociation kinetics of 0.5 kbp as well as 10 kbp long DNA fragments of the three sugarcane tissues. Table 5.1 summarizes the reassociation kinetics data of 10 kbp DNA fragments. A computer programme was allowed to find the best fit by allowing all parameters to free float. At a lower fragment size of 0.5 kbp, two distinct components, fast and slow, are distinguishable (Chapter III). However, at the higher DNA fragment size, these two fractions are merged to such an extent that their individual reassociation rates are obscured and they reassociate as a single second order component. Such a merging of repetitive DNA components when the DNA fragment length is increased is considered to be an indication of interspersed repetitive DNA components with each other.

FIGURE 5.1 REASSOCIATION KINETICS OF 10 kbp LONG FRAGMENTS OF SUGARCANE DNAs

The solid lines through the data points represent the best least squares fit, allowing all parameters to free float. The lower dotted curves represent the reassociation kinetics of pure components represents in 10 kbp long DNA.

10 kbp long DNA 0-0-0

0.5 kbp DNA - - -

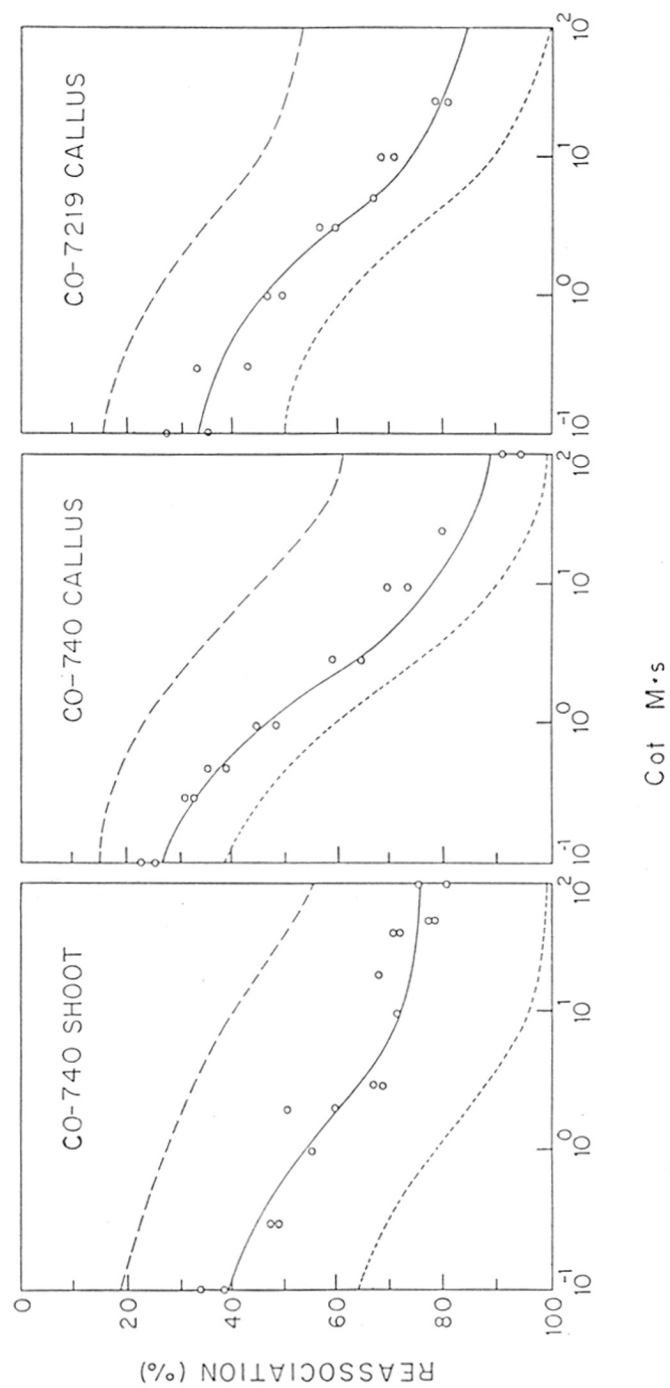


FIG. 5.1.

TABLE 5.1 : REASSOCIATION KINETICS OF LONG DNA FRAGMENTS (10 kbp)

DNA component	Fraction ^a of the genome	Cot $\frac{1}{2}$ ^a observed	K ^b observed	K predicted ^c
<u>CO-740 Callus</u>				
Fast DNA (Cot < 1 x 10 ⁻¹ M.s.)	0.26	-	-	-
Slow (Cot 1 x 10 ⁻¹ — 1 x 10 ² M.s.)	0.64	1.88	0.53	0.003
Unreassociated (Cot > 1 x 10 ² M.s.)	0.11	-	-	-
<u>CO-740 Shoot</u>				
Fast DNA (Cot < 1 x 10 ⁻¹ M.s.)	0.39	-	-	-
Slow (Cot 1 x 10 ⁻¹ — 1 x 10 ² M.s.)	0.38	1.26	0.79	0.156
Unreassociated (Cot > 1 x 10 ² M.s.)	0.24	-	-	-
<u>CO-7219 Callus</u>				
Fast DNA (Cot < 1 x 10 ⁻¹ M.s.)	0.33	-	-	-
Slow (Cot 1 x 10 ⁻¹ — 1 x 10 ² M.s.)	0.52	3.08	0.32	0.002
Unreassociated (Cot > 1 x 10 ² M.s.)	0.15	-	-	-

^aValues obtained from the respective reassociation curves (Fig. 5.1)

$$^b K = \frac{1}{\text{Cot } \frac{1}{2}}$$

^cCalculated using the formula $\frac{K1}{K2} = \frac{L1}{L2}^{\frac{1}{2}}$

where K1 and K2 are the rate constants for the reassociation of short (L1) and long (L2) fragments respectively (Wetmur and Davidson, 1968).

In order to assess the degree of interspersion in all the three sugarcane genomes, we have compared the extent of increase in HAP bound DNA of 10 kbp long DNA with that of 0.5 kbp DNA at Cot 100 (Table 5.2). In CO-740 shoot 10 kbp long DNA, the increase in HAP bound DNA is from 56% to 75% (1.33 fold) while in CO-740 callus and CO-7219 callus, it is from 61% to 86% (1.40 fold) and from 53% to 83% (1.56 fold) respectively. All the DNA reassociation values reported here have been obtained from the computer curves as shown in Figure 5.1. These data indicate that all the three sugarcane DNAs may consist of interspersed repeated and single copy DNA sequences at a fragment length of 10 kbp.

The presence of interspersion of repeated and single copy DNA sequences in sugarcane is supported by comparing the rates of reassociation observed for repetitive and single copy DNA sequences with those expected for the higher fragment size according to the formula $K1/K2 = (L1/L2)^{\frac{1}{2}}$ where L1 and L2 are the two fragment sizes under study and K1, K2 are their rate constants (Wetmur and Davidson, 1968). From Table 5.1, it can be seen that the rates observed are much different from those expected, suggesting thereby the presence of sequence interspersion in three sugarcane tissues.

It is quite likely that repeated sequences in sugarcane may show interspersion among themselves. Hence, experiments were performed to determine the HAP bound DNA at $Cot\ 1 \times 10^{-1}$.

M.s. in all the three sugarcane tissues. From the data in Table 5.2, it is clear that there is two fold increase in the amount of Cot 0.1 DNA bound to HAP in CO-740 shoot while the increase is 1.8 fold and 2.2 fold for CO-740 callus and CO-7219 callus DNAs respectively. This indicates an extensive interspersion among highly repeated DNA sequences in all the three sugarcane genomes.

S1 nuclease resistance of reassociated repetitive duplexes

Use of S1 nuclease is another approach to detect the presence of single stranded DNA stretches in repetitive duplexes isolated from the DNAs of high fragment lengths (10 kbp size). In these experiments, native DNAs (10 kbp size) of CO-740 callus, CO-740 shoot and CO-7219 callus were denatured and reassociated to Cot 100 M.s. The reassociated DNA duplexes were treated with S1 nuclease and S1 nuclease resistance repetitive DNA duplexes were isolated by HAP column chromatography as already outlined in Chapter IV. As a control, experiments were also performed without S1 nuclease treatment. The results of these experiments are summarized in Table 5.3

In case of all the three sugarcane DNAs, there is a considerable decrease in the amount of reassociated duplexes after S1 nuclease treatment. This decrease is from 86.5% to 44.2% in CO-740 callus, from 83.3% to 45.4%

TABLE 5.2: COMPARISON OF REASSOCIATION KINETICS DATA
OF 0.5 kbp AND 10 kbp LONG SUGARCANE DNAs

Species	Magnitude of increase in reassociation of 10 kbp DNA ^a	
	At Cot 1 x 10 ⁻¹ M.s.	At Cot 1 x 10 ² M.s.
CO-740 callus	1.82	1.40
CO-740 shoot	2.02	1.33
CO-7219 callus	2.27	1.56

^aCalculated from Fig. 5.1 using the formula

$$\text{Magnitude of increase in reassociation of 10 kbp DNA} = \frac{\% \text{ Reassociation of 10 kbp DNA at Cot } 1 \times 10^{-1} \text{ M.s. or at Cot } 1 \times 10^2 \text{ M.s.}}{\% \text{ Reassociation of 0.5 kbp DNA at Cot } 1 \times 10^{-1} \text{ M.s. or at Cot } 1 \times 10^2 \text{ M.s.}}$$

TABLE 5.3: S 1 NUCLEASE RESISTANT REPETITIVE DNA DUPLEXES

Source of sugarcane DNA	% Reassociated repetitive DNA duplexes without S1 nuclease treatment ^a	% S 1 resistant reassociated repetitive DNA duplexes ^b
CO-740 callus (4)	86.5 $\pm$ 4.5	44.2 $\pm$ 1.6
CO-740 shoot (4)	83.3 $\pm$ 6.1	45.4 $\pm$ 2.1
CO-7219 callus(4)	81.7 $\pm$ 1.2	48.2 $\pm$ 0.9

Figures in parenthesis indicate the number of experiments performed

a - Values obtained from experiments without S1 nuclease treatment

b - Values obtained by incubating total DNA of CO-740 callus, CO-740 shoot and CO-7219 callus tissues to Cot 100 M.s. and then isolating S1 nuclease resistant repetitive duplexes by HAP column chromatography.

in CO-740 shoots and from 81.7% to 48.2% in CO-7219 callus. Apparently this decrease in reassociation is due to digestion of single strand regions in reassociated duplexes. Since the DNA reassociation conditions were such that only repeat DNA sequences would reassociate, it is logical to assume that single strand regions must be single copy DNA sequences which remain unreassociated at Cot 100 and are contiguous to repetitive duplexes. These data thus clearly indicate the occurrence of DNA sequence interspersion in all the three sugarcane genomes.

Size distribution of reassociated repetitive DNA duplexes by Agarose A-50 column

Analysis of S1 nuclease resistant repetitive DNA duplexes by Agarose A-50 column chromatography is a direct approach to estimate their size distribution. This technique is based on the principle of gel filtration where the exclusion limit of Agarose A-50 column is 1.5 kbp. All the DNA sequences of size more than 1.5 kbp are, therefore, eluted in the void volume and those with size less than 1.5 kbp are eluted after. Calf thymus DNAs of known fragment lengths below and above the exclusion limit of Agarose A-50 were used for calibration of the Agarose column.

Figure 5.2 depicts the Agarose gel filtration profiles of S1 nuclease resistant sugarcane repetitive DNA. Table 5.4 gives the proportion of different size repetitive duplexes in the three sugarcane tissues. From this table, it can be seen that there are variations in the proportions of these fractions among the three sugarcane tissues. The proportion of fraction I (> 1.5 kbp), for example, is lowest (18%) in CO-740 callus and highest (46%) in CO-7219 callus. Similarly, the proportion of fraction II (0.2 - 1.1 kbp) is highest in CO-740 callus and lowest in Co-7219 callus.

Sizing of interspersed single copy DNA sequences

After determining the approximate size of interspersed repeat DNA sequences, the next objective was to determine the length of interspersed single copy DNA sequences. This was achieved by plotting a graph of fraction of DNA fragments binding to HAP at limit repeat Cot versus DNA fragment length.

Interspersion of repetitive and single copy DNA sequences causes a certain amount of single copy DNA to get bound to HAP at a Cot value at which only repetitive sequences are fully reassociated. Therefore, at the repetitive Cot, the amount of unreassociated single copy DNA existing as single-stranded tails on reassociated duplexes will increase as a function of DNA length. This will hold true only upto a fragment length which is equal

FIGURE 5.2 ELUTION PROFILES OF S1 NUCELEASE RESISTANT
SUGARCANE REPETITIVE DNA DUPLEXES BY AGAROSE
A-50 COLUMN CHROMATOGRAPHY

The column was developed with 0.12 M sodium
phosphate buffer (pH 6.8) and the elution
profiles were obtained by monitoring the
effluent with the help of LKB 2138
UVICORD at 253 nm.

- A - Calf thymus DNA of known duplex lengths
 used for calibration of the column
- B - CO-7219 callus
- C - CO-740 callus
- D - CO-740 shoot

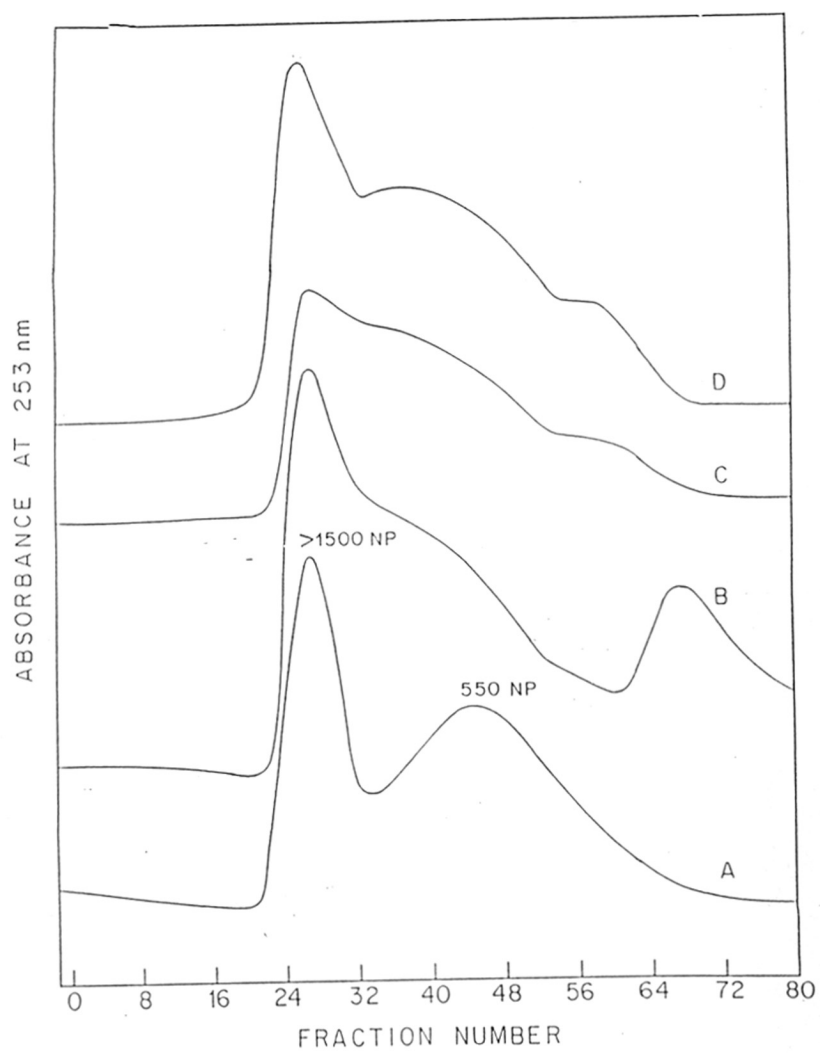


FIG. 5-2.

TABLE 5.4: PROPORTION OF DIFFERENT SIZES OF S1 NUCLEASE
RESISTANT REPETITIVE DUPLEXES IN THREE
SUGARCANE TISSUES

Species	Percent of interspersed repeats		
	Fraction I (>1.5 kbp)	Fraction II ($0.2-1.1$ kbp)	Fraction III (≤ 0.2 kbp)
CO-740 callus ⁽³⁾	18.6 ± 3.3	75.7 ± 5.0	6.0 ± 1.4
CO-740 shoot ⁽⁴⁾	35.3 ± 3.6	52.8 ± 3.1	11.7 ± 1.1
CO-7219 callus ⁽³⁾	46.3 ± 10.1	33.1 ± 10.2	20.4 ± 2.2

Figures in parenthesis indicate the number of experiments performed in each case.

Proportions of different sizes of S1 nuclease resistant repetitive duplexes were calculated by measuring the area under each peak in Fig. 5.2

to the average spacing between repetitive sequences (Wetmur and Davidson, 1968). Thus, the estimation of the average length of single copy DNA can be made by plotting a curve relating the fraction of DNA fragments bound to HAP as a function of DNA fragment length. The results of such experiments are depicted in Fig. 5.3. Two slopes are observed in all the three sugarcane tissues and the change in slope occurs at a DNA fragment length of 1.4 kbp, 1.7 kbp and 2.4 kbp for CO-740 callus, CO-740 shoot and CO-7219 callus respectively. This is the estimated average length of single copy DNA sequences in these sugarcane tissues. A more gradual increase in slope after these values indicates the presence of a wide spectrum of single copy lengths greater than 1.4, 1.7 and 2.4 kbp in CO-740 callus, CO-740 shoot and CO-7219 callus tissues, respectively.

From Fig. 5.3, it is also possible to estimate the amount of repetitive DNA in sugarcane genome. This is done by extrapolation of the line with the greater slope to the ordinate. The point of interception indicates the proportion of total repetitive DNA in each species. By doing this, the % values of duplex content of Cot 100 DNA fractions of three sugarcane tissues namely CO-740 callus, CO-740 shoot and CO-7219 callus are 42%, 56% and 48% respectively.

FIGURE 5.3 THE PERCENTAGE BINDING OF SUGARCANE REPETITIVE
DNA DUPLEXES TO HAP AS A FUNCTION OF DNA
FRAGMENTS OF DIFFERENT LENGTHS

Sugarcane DNAs were sheared to different
fragment lengths as detailed in Table 4.1,
Chapter IV. The percentage binding at
Cot 100 values was determined by hydroxy-
apatite column chromatography.

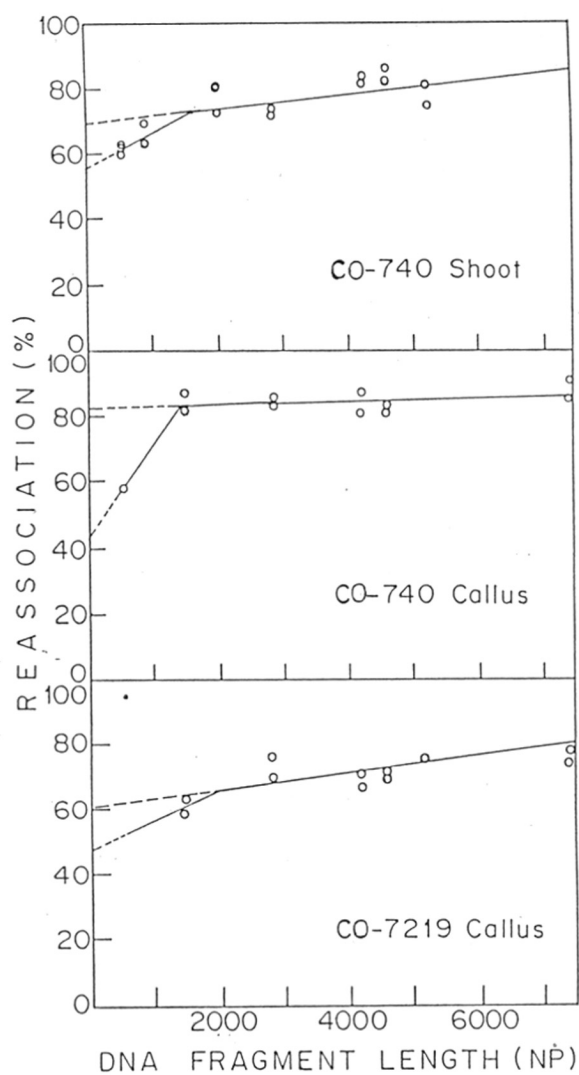


FIG. 5.3.

DISCUSSION

Sugarcane genome has a mixed pattern of DNA sequence organization

In the family Gramineae, DNA sequence organization has been studied in a few plants such as wheat (Flavell and Smith, 1976; Rimpau *et al.*, 1978), oat (Rimpau *et al.*, 1980), rye (Smith and Flavell, 1978), barley (Rimpau *et al.*, 1980), maize (Hake and Walbot, 1980), rice (Gupta *et al.*, 1981) and millets (Gupta and Ranjekar, 1981; ShivaRaman *et al.*, 1986). In maize, wheat and rye, the interspersed repeated DNA sequences are predominantly short (0.35-1.0 kbp). In little millet, barn yard millet and finger miller the interspersed repetitive sequences are both short (0.15-1.0 kbp) and long (>1.5 kbp). Pearl millet and fox tail millet have long stretches of interspersed repeat DNA sequences(>1.5 kbp). Rice is a unique plant where there is an absence of interspersion at a DNA fragment length upto 20 kbp (Gupta *et al.*, 1981, Dhar *et al.*, 1988). In case of sugarcane, ours is the first report of DNA sequence organization. Approximately 80% of the total shoot DNA consists of interspersed, repetitive and single copy DNA sequences. The interspersed repetitive DNA sequences are long (>1.5 kbp) as well as short (<0.2 kbp). Thus the interspersion pattern of sugarcane genome is of

mixed type.

According to Flavell (1980), large plant genomes (1C > 4 pg) tend to have more of their single copy DNA sequences interspersed with short stretches of repeated DNA sequences. Our earlier work on plant genome organization (Gupta et al., 1981; Gupta and Ranjekar, 1981; 1982; SivaRaman et al., 1984, 1986) has further shown that plants with 1C nuclear DNA content of less than 5 pg exhibit a mixed interspersion pattern or long interspersion pattern or even absence of interspersion pattern. Sugarcane plant has 1C nuclear DNA content of 4.0 pg (Bennett and Smith, 1976) and thus very well fits into the above trend.

Specific differences in DNA sequence organization patterns among three sugarcane tissues

Apart from studying the DNA sequence organization in short tissue, our next interest was to assess any specific differences in genome organization during in vitro differentiation in sugarcane. The three tissues namely, CO-740 callus, CO-740 shoot and CO-7219 callus resemble each other in having the presence of both long and short interspersed repeat DNA sequences in their genome. Long repeats in all the three DNAs have a heterogeneous size distribution ranging from 2.0 to 7.4 kbp. The size range of single copy DNA sequences is 1.4 - 2.2 kbp.

Thus CO-740 callus, CO-740 shoot and CO-7219 callus genomes have essentially a mixed sequence interspersion pattern of organization. A close examination of the DNA interspersion data, however, reveals at least two specific differences with respect to the extent of interspersion and the proportions of different size classes in interspersed repetitive DNA. The extent of interspersion is slightly less (75%) in CO-740 shoot DNA than that in CO-740 callus and CO-7219 callus (83-86%). Moreover, there is a significant variation in the size distribution of interspersed repetitive DNA in all the three tissues. These results thus suggest that the genome organization undergoes a few changes during in vitro differentiation in sugarcane.

Dynamics of sequence interspersion patterns

The biological significance of DNA sequence interspersion was correlated with gene regulation in eukaryotes by Britten and Davidson (1969). However, according to the later concepts (Ullrich et al., 1980; Wagenmann et al., 1981; Cullis, 1981), it appears that variations in interspersion patterns need not be correlated with gene regulation as one would not expect the basic mechanism of gene regulation to differ in various taxa. Rather, interspersion patterns should be more related to dynamics of amplification and translocation of DNA sequences (Thompson and Murray, 1980).

It has been earlier argued by Hinegardner (1976) that much of the DNA in eukaryotes is secondary and serves no strongly sequence dependent role in gene regulation. Such sequences are free to evolve by amplification, deletion and base substitution. Much of the repetitive DNA in eukaryotes is considered to originate from periodic amplification events with members of the resulting sequence families being subjected to translocation, base substitution and deletion. When both amplification and deletion occur during evolution, the portion of the genome subjected to these processes can be viewed as 'turning over' over a geological time scale. The genome size remains constant under 'steady state' conditions and the rates of amplification and deletion are equal for any one species, although different states may exist for different species. High turnover rates would lead to short period interspersed pattern as in case of maize, wheat and rye, while low turnover rates would lead to long period interspersed patterns, examples being pearl millet and fox tail millet (Wimpee and Rawson, 1979, SivaRaman, L., et al., 1986). In the present work, the fraction of sugarcane shoot genome involved in long period is 35% while that in short period interspersed is 12% (Table 5.4). The predominance of long period pattern, therefore, suggests that the sugarcane genome is turning over at a slow rate. The implications of this phenomenon are, however, not clearly known.

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CHAPTER VI

GENERAL DISCUSSION

In the present study, two main aspects of sugarcane research have been undertaken. First aspect is to develop a method for rapid multiplication of sugarcane using stem tip. This objective has been successfully achieved and it has been possible to grow the sugarcane plants in the field. It is expected that this technology could be of great value to plant breeders for propagating new sugarcane cultivars in a short period of time. The second aspect of our work is to assess the changes in DNA in callus and shoot tissues of sugarcane. The callus tissue is considered to represent a dedifferentiated stage while the shoot tissue is a differentiated one. Since the change from callus to shoot takes place under controlled experimental conditions, the callus and shoot together form an in vitro differentiating system. In the following paragraphs, we will deal with the aspect of cell differentiation since it forms a major part of the present work.

Cell differentiation is a process which involves formation of tissues and organs from proliferated cells. It covers two main aspects of differentiation, namely, in vitro differentiation induced by different hormones and in vivo differentiation. The latter covers different systems within a plant out of which the two most

extensively studied are those of embryo suspensor and and differentiating roots (Nagl 1978). The in vitro differentiation aspects have been well studied by many researchers using various molecular approaches to gain an insight into the mechanism by which an undifferentiated cell is transformed into fully differentiated tissues and vice versa. Some of the most well studied systems of in vitro differentiation include carrot, flax, tobacco Vicia faba and Cucumis melo. In these systems, the molecular parameters that have been considered to study the changes in the composition of nuclear DNA during differentiation and dedifferentiation are the determination of buoyant density, kinetic complexity, thermal denaturation behaviour and digestion patterns with restriction endonucleases. A few important findings from these data are summarized below.

In tobacco, Parenti et al. (1973) have shown the occurrence of an additional satellite DNA with a buoyant density of 1.722 g/cm^3 in pith tissue during 45-72 hrs of in vitro culture. In their further work in this direction, Durante et al. (1983) have observed amplification of G+C rich and A+T rich fractions in Nicotiana glauca pith explants in the initial hours of their in vitro culture. This amplification has been confirmed by derivative melting profiles and $\text{Ag}^+ \text{-Cs}_2\text{SO}_4$ density

gradients. These results suggest that developmental regulations may be controlled not only by differential transcription but also by specific DNA synthetic processes.

In melon (Cucumis melo), methylation level of satellite DNA sequences has been shown to be higher in hypocotyl tissue than that in callus tissue (Grisvard 1985). A similar observation has also been made in melon satellite DNA where as much as 41% of cytosine residues are replaced by 5-methyl cytosine as against 25% in main band DNA. In Vicia faba, Natali et al. (1986) have noticed the presence of a heavier satellite DNA in dedifferentiated tissue. This satellite is absent in the DNA of differentiated tissues. In carrot, extensive work has been carried out earlier by Schafer and Neumann (1978) and later by Dührssen et al. (1980). They have observed differences in reassociation and hybridization of DNA in some carrot varieties. Here, differences are in unique as well as in the repeated region of the Cot curve. Dührssen and Neumann (1980) have further reported that DNAs from callus culture and roots of the same variety deviate from each other only in highly repeated region ($Cot < 1.5 \text{ M.s.}$) of which most occurs as a heavy satellite DNA in CsCl profiles (Schafer et al. 1978, Dührssen and Neumann, 1980, Hase et al. 1979). These data indicate a differential replication of DNA sequences already present in many

copies in the carrot genome. In flax, Cullis and Cleary (1986) have compared DNAs from leaves and callus cultures of a few genotypes. They have observed that callus DNA of LH genotype has 60% more of repeated DNA sequences than the leaf DNA. The probe which is used for this comparison represents all of the highly repeated DNA sequence families in the flax genome.

In the present study, an attempt has been made to investigate molecular changes in differentiated and dedifferentiated tissues of sugarcane employing various molecular approaches. In this work, marked differences are noticed in the amount, kinetic complexity and size distribution of repeated DNA sequences in callus and shoot tissues. In CO-740 callus, for example, the fast DNA comprises 47.5% of the total DNA as against 16.5% in CO-740 shoot and 38.5% in CO-7219 callus. The kinetic complexity of the slow component of the two callus DNAs is in the range of $2.5-4.4 \times 10^8$ nucleotide pairs and is significantly higher than that in the shoot DNA (6.1×10^6 nucleotide pairs). Mini Cot curves of isolated Cot 100 DNA fraction have further indicated the presence of very fast and fast reassociating DNA components in CO-740 callus and shoot tissues and of only fast reassociating DNA component in CO-7219 callus tissue. The high resolution thermal denaturation analysis

of repetitive DNA has shown that the number of thermal components is 34, 47 and 44 in CO-740 shoot, CO-740 callus and CO-7219 callus tissues respectively. These results thus indicate that the repeated DNA sequences of the two callus tissues are more heterogeneous than that of CO-740 shoot and that there is an extensive rearrangement in repeated DNA sequences during differentiation. Another difference that is observed is in the content of 5 mC in callus and shoot DNAs in sugarcane. For example, the proportion of 5 mC is 4 moles percent for CO-740 callus and shoot DNAs and 9 moles percent in CO-7219 callus. HPLC analysis of S1 nuclease resistant repetitive DNA reveals an additional peak in CO-740 callus and shoot DNAs, the amount being 1.1 moles percent and 4.2 moles percent respectively. This additional peak is not seen in CO-7219 callus DNA. Finally, significant changes have been found in the size distribution of interspersed repetitive DNA in the three sugarcane tissues indicating that although the gross genome organization in sugarcane is of mixed type, it does undergo a change in a specific manner during differentiation.

Several theories and hypothesis have been put forward to explain the molecular events during in vitro differentiation in animal and plant tissues. We will summarize only a few of these in this paragraph.

The most widely accepted theory regarding cell differentiation holds that eukaryotic genomes remain constant in development and that differentiation is the result of a differential gene activity. Besides this, there has been an increasing speculation concerning the role of chromosomal non-histone proteins in the regulation of transcription. Later, several other groups have suggested that developmental regulation may be controlled not only by differential gene activation but also by specific DNA synthesis processes (gene amplification) at the nuclear level. The DNA sequences which are amplified during cell differentiation are not the same as those which have become underreplicated in differentiated tissues (Schafer et al. 1978, Natali et al. 1986 and Grisvard and Anghileri 1980). Kato and Tanifugi (1986) have reported that early replicating satellite DNA is probably a type or types of highly repeated DNA sequences. It may be related to regulation of genome activity to induce dedifferentiation. Duhrssen and Neumann (1980) have indicated a differential replication of DNA sequences that are already present in many copies in the carrot genome. In order to keep the balance of this preferentially synthesized DNA with total DNA, either the majority of DNA is preferentially replicated

at another period or the satellite DNA is broken down again (Schafer et al. 1978). Yet another hypothesis is that of McClintock (1978) according to whom plants have specifically evolved to adapt themselves to genetic or environmental stress. The stress hypothesis can be extended to the stress induced by in vitro culture. Indeed plant species show frequent and large variations in the relative proportions of repeated sequences after regeneration when somatic or germ cells were cultured in synthetic nutritive medium (De Paepe et al. 1982, Walbot and Cullis, 1985).

In our work, we have observed extensive genomic reorganization (which also includes underreplication) of repeated DNA sequences and marked changes in the content of 5 mC in callus and shoot DNAs of sugarcane. These findings suggest that the sugarcane DNA is dynamic and repeated DNA sequences play an important role in differentiation.

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R.R. Hendre, A.F. Mascarenhas and P.K. Ranjekar
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(India) held at Pune, November 26-28, 1983.
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(Saccharum officinarum L)
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3. Molecular basis of differentiation in sugarcane
(Saccharum officinarum L)
R.R. Hendre, A.F. Mascarenhas and P.K. Ranjekar
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Hyderabad, March 9-11 (1984).

Rapid multiplication of sugar cane by tissue culture

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Introduction

Tissue culture is now widely used in sugar cane improvement and breeding programs. This has been made possible by the pioneering work of Heinz & Mee¹ and Barba & Nickell,² who first demonstrated that plantlets could be developed from sugar cane callus cultures. Later, Heinz & Mee³ and Liu & Chen⁴ demonstrated that sugar cane plants regenerated from callus showed wide variation in chromosome number and in several important characters. This observation resulted in the introduction of tissue culture methods in sugar cane breeding programs by several countries^{5, 6}. However, a serious problem in conventional sugar cane breeding is that it takes several years before any newly isolated variety can be commercially released. Tissue culture is now being used for clonal propagation of a large number of economic plants and forest trees⁷, while Sauvaire & Galzy⁸ have recently reported a method for the vegetative propagation of sugar cane by tissue culture. No mention was made, however, of the number of plants that could be obtained on sub-culture or of the survival and growth of plants in the field. It has generally been reported that plant clones developed from meristem culture are very similar, both pheno- and genotypically, to the mother plant^{9, 10, 11}.

In this paper, we describe a method for the rapid multiplication of cane cultivar Co 740 and the successful transfer of the plants to the field. This method could be of great utility to breeders for propagating new cultivars within a short time.

Materials and methods

Virus-free sugar cane plants (*Saccharum officinarum* L. cultivar Co 740), isolated by the method described earlier^{12, 13}, were grown in the laboratory farms. Six- to

eight-month-old canes were selected and shoot apices were brought to the laboratory. Stem segments (15-20 mm) containing the meristem were excised and first rinsed with water containing a detergent, then treated with 30% of a saturated chlorine water solution for 20 minutes. At each step, the explants were washed thoroughly with sterile water. Dissection of the individual segments was then carried out in a sterile cabinet, where the leaves surrounding the meristem were removed and the shoot tip (2-3 mm) containing the meristem dome and 2-3 leaf primordia were inoculated into tubes containing 20 ml of liquid basal media. Two main media were used, one for shoot elongation and the other for shoot multiplication. That for shoot elongation (MS-I) contained mineral salts and vitamins, according to Murashige & Skoog¹⁴, supplemented with coconut water (5%), gibberellic acid (0.1 mg.l⁻¹) and indole-3-butyric acid (0.01 mg.l⁻¹); the medium for shoot multiplication (MS-II) also contained the

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13 Hendre et al.: Indian Phytopath., 1975, 28, 175-178.

14 Physiol. Plant, 1962, 15, 473-497.



mineral salts and vitamins according to Murashige & Skoog¹⁴ but was supplemented with 0.1 mg.l⁻¹ kinetin (K), 0.2 mg.l⁻¹ benzyl aminopurine (BAP) and 10% coconut water. The rooting medium contained salts and vitamins according to White¹⁵, modified by supplementation with 0.2 mg.l⁻¹ each of sodium molybdate and copper chloride. Chemicals of AnalaR grade were used for media preparation. Sucrose was added at 2% (w/v) concentration and the pH adjusted to 5.6-5.8. All media were sterilized by autoclaving at 15 psi pressure for 20 minutes and steamed for 30 minutes on the next day.

Results and discussions

Elongation of shoot tips: Shoot tips (2-3 mm) after dissection were inoculated into tubes containing 20 ml of liquid MS-I medium. As reported earlier¹³, this medium proved optimum for the growth of sugar cane apical meristems in order to obtain mosaic virus-free plants. The shoot tips were supported on filter paper stands which dipped in the liquid medium (Fig. 1a). Cultures were incubated in a growth chamber at 28°C with a 12-hour photoperiod of a light intensity of 800-1000 lux obtained from cool daylight fluorescent tubes.

and illuminated by fluorescent tubes (700 lux) for 24 hours at 30°C. In shake flasks, the shoot tips elongated further and attained a height of 40-50 mm in another 15 days. At this stage, the basal portions of leaves and the growth medium turned brown, resulting in total inhibition of growth. Removal of the brown leaves and frequent transfer of the shoots to fresh medium on alternate days for one week were found to be essential for continued viability of the shoots. These steps were essential only initially with the primary explants. By means of this treatment, the shoots survived and could later be sub-cultured regularly to fresh shake flasks containing 20 ml of MS-II medium. For the first two sub-cultures, the number of shoots obtained per flask remained the same. This rate, however, steadily increased, reaching a ratio of 1:10 after the sixth sub-culture at 15- to 20-day intervals. The ratio further increased to 1:20 and did not diminish even after 50 sub-cultures (Table I). The vigour of shoot growth did not decrease, even after 50 passages.

Multiple shoots in flasks were healthy but did not develop roots (Fig. 2). For root initiation, shoots were separated individually and inoculated into tubes containing 20 ml of rooting medium and supported on a filter paper platform. Within 15 days, 80% of the shoots developed a healthy root system with root hairs; a few plants also developed tillers. Rooted plants were washed with water to remove adhering medium and then transferred to pots containing a 1:1 mixture of sterile soil and vermiculite. Individual plants were covered

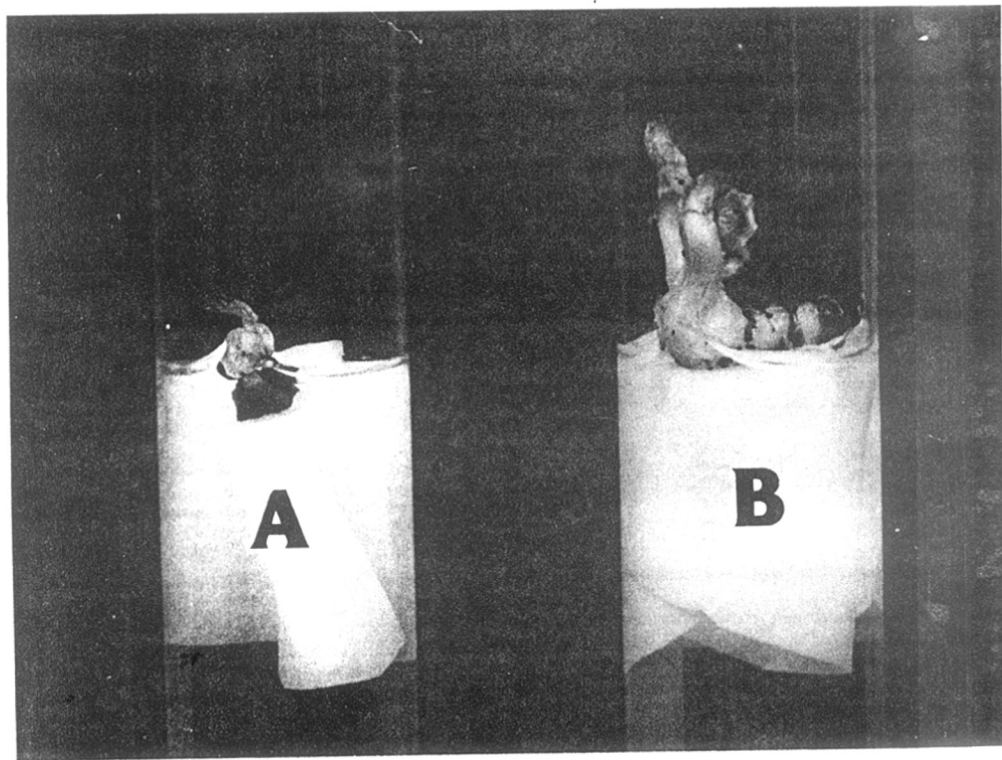


Fig. 1. (a) Initial size of shoot tip; (b) Shoot tip elongated (20 days)

Multiplication of shoots: 75-80% of the cultures elongated and attained a height of about 25 mm in 15-20 days (Fig. 1b). At this stage, individual elongated shoot tips were transferred to conical flasks containing 20 ml of liquid MS-II medium. The flasks were held in a rotary shaker (120 rpm)

with beakers to maintain humidity and were kept for hardening at 25°C under light of 800-1000 lux intensity for two weeks. They were then transferred to the glasshouse.

¹⁵ "The cultivation of animal and plant cells" (Ronald Press, New York) 1963.

Table I. Rate of multiplication of sugar cane

Sub-culture No.	No. of shoots cultured	No. of shoots recovered	Multiplication rate	Time interval per passage, days
1	30	30	1:1	15
2	18	18	1:1	15
3	16	65	1:4	15
4	8	33	1:4	15
5	12	110	1:9	15
6	15	148	1:10	15
7	22	266	1:12	15
8	18	280	1:15	15
9	14	249	1:18	15
10	23	465	1:20	15

Medium: MS-II
Inoculum: 1 shoot per flask

When they had grown to a height of 75-100 mm, they were transferred to the field, where their survival was 80%. These conditions for obtaining shoot and root formation were repeated four times using fresh explants, and found to be reproducible.

As shown in Table II, on the basis of the rate of multiplication at each sub-culture, it is possible to produce nearly 260,000 shoots from a single shoot tip in 4 months after the first sub-culture. If all these shoots are kept in rooting medium, 200,000 rooted plants can be obtained in 15 days (allowing for rooting of 80% of the shoots). These

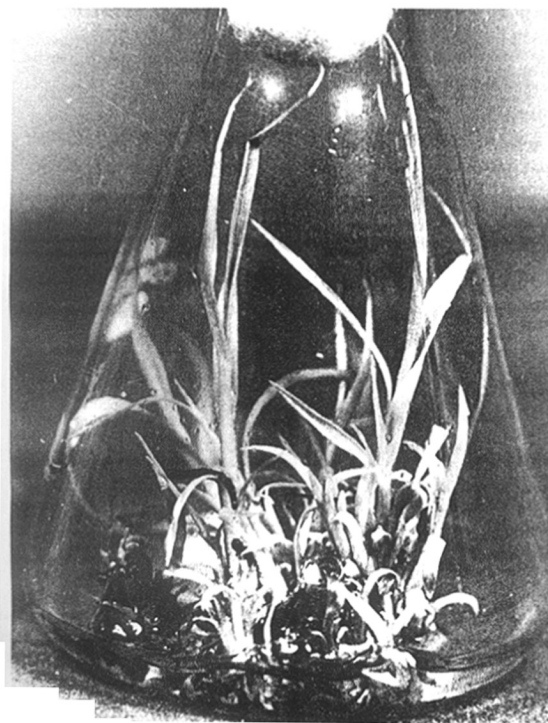


Fig. 2. Multiple shoot formation in shake flask

Rapid multiplication of sugar cane

rooted plants can then be transferred to pots containing a mixture of soil and vermiculite for hardening, which takes about 2-4 weeks. Thus, about 200,000 plants can be obtained in 6 months, which is sufficient for planting 10 ha at a population density of 20,000 plants per ha.

Table II. Scale-up for producing 200,000 plants

Step No.	No. of shoots inoculated	Rate of multiplication	No. of shoots recovered	Period from beginning
1	1	1:1	1	15 days
2	1	1:1	1	1 month
3	1	1:4	4	1 month
				15 days
4	4	1:4	16	2 months
5	16	1:9	144	2 months
				15 days
6	144	1:10	1,440	3 months
7	1,440	1:12	17,280	3 months
				15 days
8	17,280	1:15	259,200	4 months

Damiano¹⁶ reported that, in order to produce 200,000 strawberry plantlets *in vitro* per year, two skilled technicians are necessary. For transfer of plantlets to pots and then further hardening, one more unskilled worker will be needed. The space requirements for producing 200,000 plants per year

is 165 m², including rooms for the laboratory and incubation of cultures. The equipment, glassware and chemicals required for such a laboratory have also been described. A similar arrangement can be used for producing the sugar cane plants, since the rates of multiplication in both cases are more or less identical.

In the method described by Sauvaire & Galzy⁸, axillary buds of sugar cane were used; addition of kinetin and NAA to Murashige & Skoog's basal medium favoured tillering. Their studies were continued for only four sub-cultures by separation and reinoculation of tillers. No details are available on the multiplication rates or on survival of the plants in the field. The conditions of growth used in the present studies differ greatly from those used by Sauvaire & Galzy; a sequence of transfers is required before viable, healthy and vigorously growing multiple shoots can be obtained. These plants have also been grown in the field (Fig. 3).

Field trials

Over 1000 clonal plants were obtained and planted in the field. To determine variation, if any, in clonal plants produced by tissue culture, observations were made on 100 plants grown in a 11 × 11 m plot and spaced 1 m apart. Table III shows the average results of this trial. The heights of the plants, number of internodes per cane, weights of individual canes and the number of canes per clump were more or less the same for all plants, with very little deviation from the average values.



Fig. 3. Sugar cane plants from tissue culture in field

These results clearly demonstrate the applicability of the method for large-scale propagation of new sugar cane varieties in a short period of time. This method could be highly valuable in sugar cane breeding programs for rapid multiplication of any newly released varieties, which could take several years using conventional methods.

Perspectives and new developments for agrochemicals in sugar cane

(continued from page 4)

cables, or furthermore may arise indirectly through the transmission of diseases on the estate.

Losses are due not only to the physical damage but, perhaps more importantly, to fermentation of the sugar in damaged stalks, secondary invasion by diseases (particularly red core) and general loss of vigour.

Up until now there have been no really satisfactory chemicals for rodent control in sugar cane. Multiple feed anticoagulants are only practical for perimeter baiting, which gives little or no control of rodents living in the mature crops. Applications with a high volume of bait and labour are also expensive. Traditional acute poisons such as zinc phosphide are much more practical (e.g. application from aircraft over the crop), but show severe problems of bait shyness. Because the bait is thinly scattered, the rodents can become shy of the bait before they find a lethal dose.

There is now increasing evidence that the new ICI rodenticide Klerat, based on Brodifacoum, will be the first product to achieve really effective results in sugar cane as well as being practical to apply. This new compound is broad-

Table III. Field data on clonally propagated sugar cane plants

Plot size (m)	11 × 11
Spacing (m)	1 × 1
Date of planting	June 16, 1980
Date of harvest	Sept. 30, 1981
Number of plants sown	100
Number of plants surviving	83
Height of millable canes (m)	2.64±0.024
Number of canes/clump	18±0.023
Number of internodes/cane	30±0.23
Weight of individual canes (kg)	1.124±0.024
Number of canes per plot	1494

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Summary

Shoot tips of sugar cane cultivar Co 740 were cultured on a basal medium supplemented with growth regulators where they elongated. These elongated shoot tips were transferred to a second medium supplemented with cytokinins on which 15-20 shoots were produced from a single shoot tip within 2-3 weeks. Rooting was induced by separating the individual shoots and transferring them to a simple medium without any hormones. Within 2 weeks, 80% of the plants developed a healthy root system. By means of transfers at regular intervals, it is possible to produce about 200,000 plants in

6 months from a single shoot tip. These plants have been successfully grown in the field. Field data show that clonally propagated plants show very little deviation in the length of millable canes, number of internodes and the number of canes per clump. This method could permit cane breeders to multiply new cultivars in a short period of time.

spectrum and lethal from a single feed, leading to a low rate of use per hectare. Moreover, there is no evidence of poison shyness and no plant uptake. Apart from efficacy tests, work is continuing on formulations and application techniques. More practical systems of monitoring are also being developed so that estate managers can routinely check whether rodenticides should be applied.

Conclusions

This paper has attempted to set in perspective the usage of agrochemicals in sugar cane, relative to other major crops, and illustrates that sugar cane represents a relatively small component of the total. As a specific crop it is thus, in broad terms, likely to benefit from new agrochemicals more as a result of "spin-off" activity rather than from specifically targeted research for totally new compounds.

Nevertheless, it has been possible to point to a number of new and interesting developments for agrochemicals in sugar cane. These have resulted, to a significant degree, from the work of many excellent sugar cane research stations around the world as well as from the research and development statistics of the agrochemical companies.

Differences in Repeated DNA Sequences Between Callus and Shoot Tissue of Sugarcane (*Saccharum officinarum* L)*

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Distinct differences have been noticed in the amount, kinetic complexity and size distribution of repeated DNA sequences in callus and shoot tissues of sugarcane. The proportion of total repetitive DNA is 62% in the callus Co-740 and 33% in Co-740 shoots. The kinetic complexity of the fast reassociating DNA in the callus tissue is forty-times that in shoots and the proportion of long repeats (> 1500 bp) is more in shoots than that in Co-740 callus tissue, thus suggesting that repeated DNA sequences undergo a significant change during differentiation of callus tissue to shoots.

It is now generally accepted that eukaryotic DNA is dynamic and undergoes selective rearrangements and changes during cell differentiation. In plants variations in complexity and composition of nuclear DNA have been reported during cell differentiation¹⁻⁶. Differential replication of satellite DNA and main band DNA has also been described during dedifferentiation in carrot⁷ and melon⁸. The rDNA, however, has been shown to undergo no gross change during dedifferentiation in carrot and Jerusalem Artichoke explants⁹.

Plant tissue culture offers unique systems for studying plant genomes under controlled conditions. We have been interested in sugarcane system because of the following reasons: (i) sugar cane callus (dedifferentiated) tissue could be readily obtained and maintained by regular subculturing, (ii) differentiation could be easily induced in callus cultures by omitting auxin from the medium and incubating in the presence of light (12 hr/day), (iii) multiple shoots without roots could be propagated vegetatively by *in vitro* techniques.

In our laboratory, extensive tissue culture work is being carried out on the two sugarcane varieties, namely Co-740 and Co-7219. The former variety has retained the capacity for differentiation even after ten years of its subculturing, whereas the latter variety has lost its capacity to differentiate even within one year of subculturing. We have, therefore, isolated DNAs from these two callus varieties and shoots obtained from

Co-740 and have characterized them with an objective to assess the changes in the gross composition of DNA in callus and shoot tissues of sugarcane. In this paper, we present the following data:

- (1) Non-linear least-squares regression analysis of the DNA reassociation kinetics.
- (2) Thermal denaturation and optical reassociation of repetitive DNA fractions.
- (3) Size fractionation of SI nuclease-resistant repetitive DNA.

Materials and Methods

Isolation and maintenance of tissues—Sugarcane plants of two varieties (Co-740 and Co-7219) were grown in the experimental farms of National Chemical Laboratory, Pune. Stem tips (about 2 cm long) were excised from 6-8 months old plants and were surface sterilized using chlorine water¹⁰. Segments (0.5 - 1.0 cm) of the innermost young leaf, adjacent to the meristem, were cultured on a semisolid medium containing salts and vitamins according to Murashige and Skoog¹¹ and supplemented with coconut milk (10%, vol./vol.), inositol (20 mg/l) and 2,4-dichlorophenoxy acetic acid¹² (3 mg/l). Callus was formed within 15-20 days and was subcultured into a fresh medium every month.

Stem tips of sugarcane (variety Co-740, 2-4 mm long) containing 2 - 3 leaf primordia were cultured on liquid medium containing salts and vitamins according to Murashige and Skoog¹¹ and supplemented with coconut milk (5%, vol./vol.), gibberellic acid (0.1 mg/l) and indolebutyric acid (0.01 mg/l). The cultures were incubated at 28°C with a photoperiod of 12 hr/day. Within 15 days, stem tips were elongated to about 2 cm.

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By repeated subculturing of elongated stem tips in liquid medium containing salts and vitamins according to Murashige and Skoog¹¹ and supplemented with coconut milk (10% vol./vol.), kinetin (0.1 mg/l) and benzylaminopurine (0.2 mg/l), multiple shoots without roots were formed. Shoots were subcultured into fresh medium every 15 days¹³.

Isolation of DNA—Callus tissues (21-day-old) and multiple shoots (15-day-old) were frozen in liquid nitrogen and stored at -20°C till further use. DNA was extracted from the frozen tissue using the method of Bendich and Bolton¹⁴. Approx. 100g of frozen tissue was first powdered in a prechilled mortar followed by homogenization in a mixer for 1-2 min at high speed. About 5-10 mg DNA was obtained from 100 g of the frozen tissue. The purified DNA preparations showed OD_{280/260} of 0.55 and OD_{230/260} of 0.45 and were also free from detectable levels of polysaccharide contamination. All the preparations exhibited approx. 24% hyperchromicity upon thermal denaturation in 0.12M sodium phosphate buffer (pH 6.8).

Isopycnic centrifugation of DNA in neutral CsCl gradient—Analytical equilibrium ultracentrifugation in neutral CsCl gradient was performed in a Spinco Model E ultracentrifuge equipped with UV scanner. DNA samples (5-10 μg) were centrifuged for 30 hr at 36,000 rpm at 25°C . The buoyant densities were calculated using *Micrococcus lysodeikticus* DNA ($\rho = 1.731 \text{ g/cm}^3$) as a marker DNA¹⁵ and base compositions of DNAs were calculated from their respective buoyant densities¹⁶.

Sonication, denaturation and reassociation of DNA—DNA was fragmented to an average size of 475-550 bp using Biosonic III sonicator fitted with 1.25 cm probe¹⁷. The sonicated DNA was denatured by heating in boiling water-bath for 10 min and reassociated in 0.12 M sodium phosphate buffer (pH 6.8) at 62°C according to Britten *et al.*¹⁸. Thermal denaturation and optical reassociation experiments were performed using Gilford 250 spectrophotometer, equipped with an analog multiplexer (model 6064), thermoprogrammer (model 2527) and a reference compensator¹⁹. The melting temperature (T_m), G+C content, hyperchromicity and σ 2/3 were calculated according to the standard procedures²⁰⁻²³. The DNAs from mouse and *E. coli* served as controls²⁴⁻²⁶.

Isolation and sizing of SI nuclease-resistant repetitive duplexes—In order to determine the length of repeated DNA sequences, native, unsonicated DNAs (> 20 kilo base pairs in length) were reassociated to Cot 100 in 0.18 M NaCl containing 0.006 M PIPES (pH 6.8). After reassociation, the DNA samples were adjusted to 25 mM sodium acetate (pH 4.5)/0.1 mM ZnSO_4 /5 mM

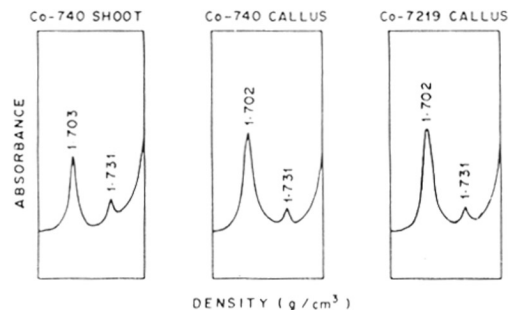


Fig. 1. Scan showing the isopycnic band of sugarcane DNA in neutral CsCl density gradient after reaching equilibrium. [Initial density of CsCl was 1.7000 g/cm^3 . The sample was spun at 36,000 r.p.m. for 30 hr. *Micrococcus lysodeikticus* DNA was used as a reference marker]

2-mercapto ethanol²⁷ and treated with SI nuclease (10 units/ μg DNA). The SI nuclease-resistant duplexes were isolated by hydroxyapatite column chromatography and dialysed against 0.12 M sodium phosphate buffer (pH 6.8).

The size distribution of SI nuclease-resistant repetitive duplexes was determined by gel filtration on Agarose A-50 column²⁸ (100-200 mesh, Biorad Laboratories).

Results

Buoyant density of DNA—As seen in Fig. 1, the DNA from the two callus tissues and shoot tissue banded as a single narrow peak without any shoulder or hump on its light or heavy side. The buoyant density of the DNA from both the callus tissues was 1.702 g/cm^3 . Since the chloroplast and mitochondrial DNAs generally have buoyant density of 1.697 g/cm^3 and 1.706 g/cm^3 respectively²⁹, it can be concluded that the DNAs under consideration are mainly of nuclear origin. From the buoyant density, the G + C content of all the DNAs is estimated to be 42.85-43.87%.

Nonlinear regression analysis of Cot curves—Fig. 2 depicts the reassociation curves of the DNAs from two callus cultures, namely Co-740 and Co-7219, and shoots of Co-740. Non-linear least squares regression analysis of the curves reveals the presence of one fast and one slow DNA component each reassociating with second order kinetics (Table I) in all the three systems studied. A comparison of the rate constants of the two DNA components indicates that the fast reassociating DNA fraction consists of repeated DNA sequences while the slow reassociating DNA fraction is made up of mainly single copy sequences. Marked differences are, however, noticed among the three systems in respect of the proportion, frequency of reiteration and

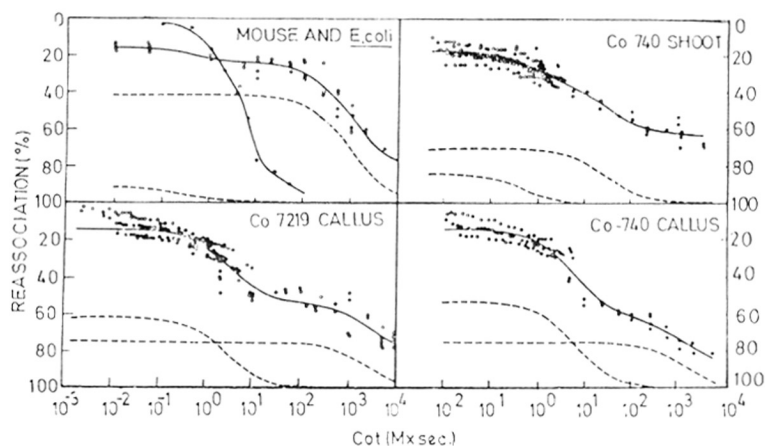


Fig. 2—Reassociation curve of 550 bp long DNAs of Co-740 shoot, Co-740 callus and Co-7219 callus obtained using optical spectrophotometry (Cot values up to $5 M \times \text{sec./litre}$) as well as hydroxyapatite column chromatography (Cot values in the range of $0.1 - 5 \times 10^3 M \times \text{sec./litre}$). [The DNA in $0.12 M$ sodium phosphate buffer ($pH 6.8$) was denatured at $100^\circ C$ and incubated at $62^\circ C$ to obtain desired Cot values. The solid line through the data points represents the least squares fit for the components allowing all the parameters to free float. The lower dashed curves represent the reassociation kinetics of pure components. Mouse DNA ($\circ-\circ$) and *E. coli* DNA ($\bullet-\bullet$) were used as standard]

Table 1—Reassociation Kinetics of Sugarcane

DNA fraction	Fraction ^a of DNA	Cot $\frac{1}{2}$ ^a observed $M \times \text{sec./litre}$	Frequency of repetition	Cot $\frac{1}{2}$ ^b pure $M \times \text{sec./litre}$	Kinetic complexity ^d n.p.
<i>Co-740 Callus</i>					
Fast reassociating DNA (Cot $10^{-2} - 10^2$ $M \times \text{sec./litre}$)	0.47	6.40	2.09×10^2	3.04	2.28×10^6
Slow reassociating DNA (Cot $10^2 - 5 \times 10^3$ $M \times \text{sec./litre}$)	0.25	1.34×10^3	1	3.43×10^2	2.57×10^8
<i>Co-740 Shoots</i>					
Fast reassociating DNA (Cot $5 \times 10^{-3} - 2.5$ $M \times \text{sec./litre}$)	0.16	4.64	5.80×10^1	7.63×10^{-2}	5.70×10^4
Slow reassociating DNA (Cot $2.5 - 2 \times 10^3$ $M \times \text{sec./litre}$)	0.30	2.71×10^1	1	8.16	6.12×10^6
<i>Co-7219 Callus</i>					
Fast reassociating DNA (Cot $10^{-2} - 10^2$ $M \times \text{sec./litre}$)	0.38	3.11	7.26×10^2	1.19	8.99×10^5
Slow reassociating DNA (Cot $10^2 - 5 \times 10^3$ $M \times \text{sec./litre}$)	0.25	2.26×10^1	1	5.84×10^2	4.38×10^8

^a, values obtained from the respective reassociation curves (Fig. 2) [The proportion of DNA in Co-740 callus, Co-740 shoots and Co-7219 callus, was respectively 0.14, 0.16 and 0.14 for the very fast reassociating DNA fraction, (Cot $< 10^{-2} M \text{ sec./litre}$ for callus and Cot $< 5 \times 10^{-3} M \text{ sec./litre}$ for shoots) and 0.12, 0.37 and 0.20 for the 'Unreassociated DNA fraction' (Cot $> 5 \times 10^3 M \text{ sec./litre}$ for callus and Cot $> 2 \times 10^3 M \text{ sec./litre}$ for shoots). ^b, Cot $\frac{1}{2}$ values of unique DNA divided by Cot $\frac{1}{2}$ values of the given fraction; ^c, Cot $\frac{1}{2}$ values observed $\times$ fraction of genome; ^d, values obtained by comparing the Cot $\frac{1}{2}$ under our experimental conditions and genome size 4.5×10^6 bp of *E. coli*²⁶.

kinetic complexity of the fast reassociating DNA component. In Co-740 callus, for example, the fast DNA comprises 47.5% of the total DNA as against 16.5% in shoot and 38.5% in Co-7219 callus. The frequency of repetition and kinetic complexity of the DNA sequences in this fraction is also high in callus culture as compared to those in shoots. The proportion of the slow component is 25 - 30% in all the three systems. Its kinetic complexity is in the range of $2.5 - 4.4 \times 10^8$ np in both the types of callus cultures and is significantly higher than that of shoot DNA (6.1×10^6 np). Apart from the fast and slow reassociating DNA components, 14 - 16% of the total DNA forms

duplexes very rapidly at $Cot < 10^{-2}$ M-sec/liter and 12 - 37% of the total DNA fails to reassociate at $Cot > 10^3$ M-sec/liter. Thus the total proportion of the repetitive DNA is 62% in Co-740 callus, 33% in Co-740 shoots and 53% in Co-7219 callus.

Analysis of mini Cot curves—To assess the kinetic heterogeneity of repetitive DNA fractions in the three systems, mini Cot curves of isolated Cot 100 DNA fractions were obtained. The data depicted in Fig. 3 and summarized in Table 2 indicate the presence of very fast and fast reassociating DNA components of Co-740 callus and Co-740 shoots and only fast reassociating DNA component in Co-7219 callus. The

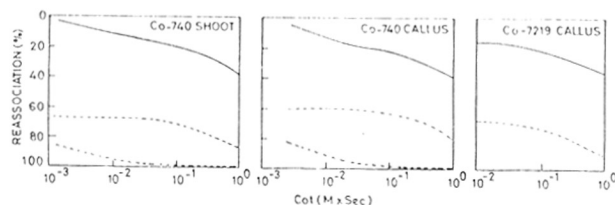


Fig. 3—Optical reassociation curve of Cot 100 fraction of Co-740 shoots, Co-740 callus and Co-7219 callus in 0.12 M sodium phosphate buffer (pH 6.8) [After denaturation at 98 °C, the temperature of cuvette block was lowered to 62 °C and the drop in optical density of each DNA was recorded as a function of time. The zero time was considered at 80 °C according to Britten *et al.* (1974). Percent reassociation was calculated from the drop in optical density and Cot values were derived from the time of incubation and DNA concentration]

Table 2—Reassociation Kinetics of Isolated Cot 100 DNA Fractions

DNA fraction	Fraction ^a of DNA	Cot 1/2 ^c observed M × sec/litre	Frequency of ^b repetition	Cot 1/2 ^c pure M × sec/litre	Kinetic complexity ^d n.p.
<i>Co-740 Callus</i>					
Very Fast reassociating DNA (Cot 2.6×10^{-3} - 2.6×10^{-1} M × sec/litre)	0.17	5.45×10^{-3}	2.45×10^5	1.37×10^{-3}	1.02×10^3
Fast reassociating DNA (Cot 2.6×10^{-1} - 1.0 M × sec/litre)	0.39	9.90×10^{-1}	1.35×10^3	3.90×10^{-1}	2.92×10^5
<i>Co-740 Shoots</i>					
Very fast reassociating DNA (Cot 3×10^{-3} - 1×10^{-1} M × sec/litre)	0.17	3.60×10^{-3}	7.54×10^3	6.24×10^{-4}	4.68×10^2
Fast reassociating DNA (Cot 1×10^{-1} - 1.0 M × sec/litre)	0.33	6.57×10^{-1}	4.13×10^1	2.12×10^{-1}	1.63×10^5
<i>Co-7219 Callus</i>					
Fast reassociating DNA (Cot 9×10^{-3} - 1.0 M × sec/litre)	0.32	4.64×10^{-1}	4.80×10^3	1.48×10^{-1}	1.11×10^5

^a, values obtained from the respective mini Cot curves (Fig. 3) [The proportion of DNA in Co-740 Callus, Co-740 shoots and Co-7219 callus was, respectively, 0.03, 0.03 and 0.17 for the 'Instantaneously reassociating DNA fraction' (Cot $< 2.6 \times 10^{-3}$ M sec/litre for Co-740 callus, Cot $< 3 \times 10^{-3}$ M sec/litre for shoots and Cot $< 9 \times 10^{-3}$ M × sec/litre for Co-7219 callus) and 0.40, 0.50 and 0.52 for the 'Unreassociated DNA fraction' (Cot > 1 M sec/litre for Co-740 callus, shoots and Co-7219 callus)]; ^b, Cot 1/2 values of unique DNA divided by Cot 1/2 values of the given fraction; ^c, Cot 1/2 values observed × fraction of genome; ^d, values obtained by comparing Cot 1/2 under our experimental conditions and genome size 4.5×10^6 base pairs of *E. coli*²⁶.

Table 3—Thermal Denaturation Properties of Native and Repetitive DNA Fractions

1	Reassociation (%)	Hyperchromicity (%)	Melting temperature (T _m °C)	G + C (%)	σ 2/3 (°C)
2	3	4	5	6	
<i>Co-740 Callus</i>					
Native unsonicated (10)	—	23.49 ± 0.57	86.48 ± 0.24	42.06 ± 0.66	7.9 ± 0.12
Native sonicated (550 np) (5)	—	20.23 ± 4.80	85.12 ± 0.39	—	9.08 ± 0.57
Cot 0.1 fraction (4)	29.42 ± 6.75	8.52 ± 1.05	Low melting 78.6 ± 0.20 High melting 92.4 ± 1.80	—	—
Cot 0.1 - 10 fraction (4)	46.69 ± 0.95	12.4 ± 1.16	76.55 ± 0.90	—	—
Cot 100 fraction (7)	60.28 ± 2.95	14.97 ± 2.84	Low melting 77.05 ± 0.61 High melting 93.2 ± 0.41	—	—
Cot 100-500 fraction (4)	35.38 ± 5.83	11.31 ± 3.30	76.95 ± 0.83	—	—
<i>Co-740 Shoots</i>					
Native unsonicated (7)	—	24.17 ± 1.72	86.2 ± 0.30	41.23 ± 1.83	8.34 ± 0.18
Native sonicated (550 np) (8)	—	17.43 ± 5.60	84.57 ± 0.58	—	9.63 ± 0.83
Cot 0.1 fraction (4)	23.72 ± 0.24	9.07 ± 2.53	Low melting 78.86 ± 0.28 High melting 96.0 ± 0.40	—	—
Cot 0.1 - 10 fraction (4)	37.47 ± 5.91	14.82 ± 1.55	75.95 ± 0.44	—	—
Cot 100 fraction (5)	63.95 ± 0.48	15.20 ± 2.77	Low melting 75.86 ± 0.57 High melting 93.1 ± 0.11	—	—
Cot 100 - 500 fraction (4)	30.44 ± 2.44	9.08 ± 0.78	74.95 ± 0.77	—	—
<i>Co-7219 Callus</i>					
Native unsonicated (8)	—	24.02 ± 0.73	86.65 ± 0.08	42.33 ± 0.62	8.07 ± 0.17
Native sonicated (550 np) (6)	—	19.88 ± 0.54	84.53 ± 0.54	—	9.6 ± 0.83
Cot 0.1 fraction (4)	25.24 ± 1.50	7.95 ± 0.04	Low melting 77.0 ± 0.6 High melting 93.95 ± 0.15	—	—
Cot 0.1 - 10 fraction (4)	43.32 ± 1.18	14.43 ± 3.60	74.9 ± 0.70*	—	—
Cot 100 fraction (8)	50.67 ± 1.50	17.11 ± 1.76	Low melting 75.75 ± 0.70 High melting 93.0 ± 0.87	—	—
Cot 100 - 500 fraction (4)	20.16 ± 0.38	8.76 ± 1.89	77.4 ± 0.20	—	—

Numerals in parentheses indicate the number of experiments.

frequency of reiteration and the kinetic complexity of the very fast reassociating DNA component are 2.4×10^5 and 1.0×10^3 np respectively, in Co-740 callus and 7.5×10^3 and 4.7×10^2 np, respectively, in Co-740 shoots. The fast reassociating DNA component, present in all the three systems, has a similar kinetic complexity of $1.1 - 2.9 \times 10^5$ np and frequency of repetition in the range $4.1 \times 10^1 - 4.8 \times 10^3$.

Thermal denaturation studies—The total DNAs and the repetitive DNA fractions were next characterized by studying their melting properties. From Fig. 4 and Table 3, it can be seen that the melting temperatures and hyperchromicities of the total DNAs in all the three systems are similar. The G + C content values of the DNAs estimated from their T_m values compare reasonably well with those obtained from the buoyant density values.

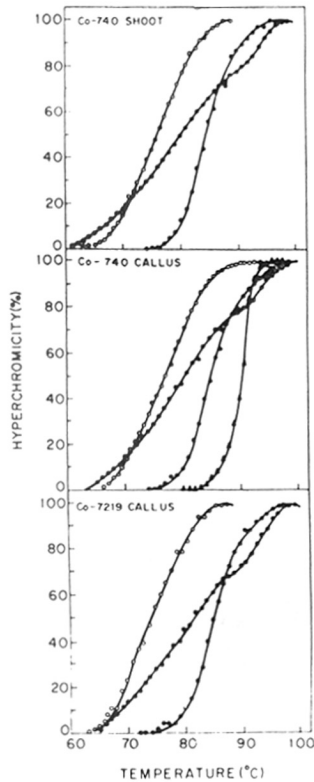


Fig. 4—Thermal denaturation profiles of sonicated DNAs (○ — ○) Cot 0.1 - 10 fractions (○ — ○) and Cot 100 DNA fractions (● — ●) of Co-740 callus with *E. coli* DNA (△ — △) as standard. Co-740 shoots and Co-7219 callus [For isolating Cot fractions, Cot 100 DNA was denatured, reassociated to Cot 0.1 and fractionated on hydroxyapatite column. DNA fraction eluted with 0.12 *M* sodium phosphate buffer (pH 6.8) was again denatured, reassociated to Cot 10 and fractionated on hydroxyapatite column. The reassociated DNA fraction was eluted with 0.4 *M* sodium phosphate buffer (pH 6.8). All melting experiments were carried out in 0.12 *M* sodium phosphate buffer (pH 6.8). The temperature of the DNA was raised at a rate of 1°C min]

Analysis of the Cot fractions has, however, yielded interesting results. Cot 0.1, 10 and 100 DNA fractions in all the three DNA systems have biphasic melting profiles with a major low melting (T_m 75.7 - 78.9°C) and a minor high melting (T_m 92.4 - 96.0°C) DNA component. The DNA fractions like Cot 0.1 to 10, 10 to 100 and 100 to 500 show smooth, monophasic melting profiles indicating that the high melting component is restricted to Cot 0.1 DNA only in all the three systems. Melting profiles of Cot 100 and Cot 0.1 to 10 of all the three systems are depicted in Fig. 4 as representatives.

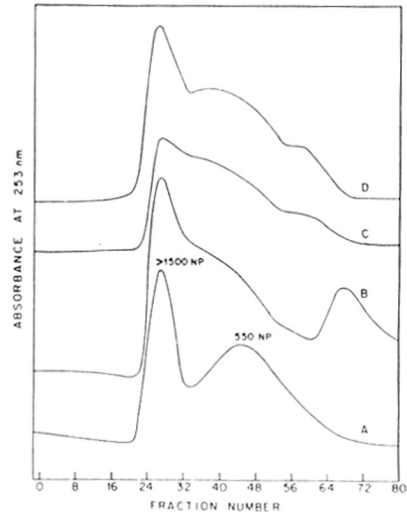


Fig. 5 - Sizing of SI nuclease-resistant repetitive duplexes isolated at Cot 100 by Agarose A-50 column chromatography. [Calf thymus DNA of known duplex lengths were used for the calibration of the column. The column was developed with 0.12 *M* sodium phosphate buffer (pH 6.8) and elution profiles were obtained by monitoring the effluent with the help of an LKB UVICORD at 253 nm. (A), calf thymus; (B), Co-7219 callus; (C), Co-740 callus; (D), Co-740 shoot]

Table 4—Analysis of Agarose A - 50 Column Chromatography Profiles of SI-Resistant Repetitive DNAs

	> 1500 np (%)	700-1100 np (%)	< 200 np (%)
Co-740 Shoots (4)	35.3 ± 3.6	52.8 ± 3.1	11.7 ± 1.1
Co-740 Callus (3)	18.6 ± 3.3	75.7 ± 5.0	6.0 ± 1.4
Co-7219 Callus (3)	46.3 ± 10.1	33.1 ± 10.2	20.4 ± 2.2

Numerals in parantheses indicate the number of experiments.

Size distribution of SI-resistant repetitive duplexes—

From Fig. 5 and Table 4, it is clear that there are three distinct size classes of repetitive sequences in all the three systems. The proportions of three classes are, however, different. The proportion of DNA duplexes of size > 1500 np is, for example, 35% in Co-740 shoot DNA, 18% in Co-740 callus DNA and 46% in Co-7219 callus DNA. Significant differences are also observed in the proportion of the DNA duplexes that have a size range of 700-1100 np and < 200 np

Discussion

DNA reassociation kinetics (Cot analysis) is one of

the most powerful approaches to study genome organization in eukaryotes. It gives an overall information of the distribution, percentage and average size of repetitive and single copy DNA components. Non-linear regression analysis of the reassociation data further helps to resolve the Cot curves into repetitive and single copy components. Using this approach, it has been possible to arrive at the following specific differences with respect to the proportion and size distribution of repeated DNA sequences in sugarcane in callus and shoot tissues.

(i) The content of repetitive DNA is two fold more in Co-740 callus than that in Co-740 shoots.

(ii) Repetitive DNA consists of only one kinetic fraction in Co-7219 callus as against two fractions in Co-740 callus and shoots.

(iii) The size distribution of DNA sequences in the SI nuclease resistant repetitive DNA fraction is characteristic for callus and shoot tissues.

A question can be raised whether the observed variations in repetitive DNA content between callus and shoot tissues of sugarcane are due to the presence of chloroplast and mitochondrial DNAs. The mitochondrial DNA generally accounts for less than 1% of the total DNA and hence its effects can be considered as insignificant³⁰. The chloroplast DNA represents up to 10% of the total genome in green tissues³¹. The proportion of repetitive DNA which may also include chloroplast DNA in shoot tissue is, however, half of the repetitive DNA present in callus tissue. It is, therefore, clear that the significant differences in the content of repetitive DNA in callus and shoot tissues cannot be due to the presence of organelle DNA.

The precise significance of the differences in repeated DNA sequences between the two callus tissues and between callus and shoot tissues cannot be explained at this stage. It can be pointed out, however, that repeated DNA sequences undergo a considerable change in their proportion, kinetic complexity and size distribution during the transition from callus tissue to shoot tissue in sugarcane.

Our cytological studies in family Leguminosae have already indicated that there is an under-replication of heterochromatin during *in vivo* differentiation^{32,33}. The present molecular data in sugarcane also shows a marked diminution in the proportion of repeated DNA sequences during the change of callus tissue to shoot tissue. This is a very interesting observation and further detailed work involving analysis of repetitive DNA duplexes using restriction endonucleases is now undertaken.

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