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CHAPTER 6
SUGARCANE - PLANT REGENERATION AND DNA ANALYSIS OF REGENERATED PLANTS

CHAPTER VI - SUGARCANE - PLANT REGENERATION AND DNA ANALYSIS OF REGENERATED PLANTS.

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- Plant acclamatization
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INTRODUCTION

In vitro regeneration of sugarcane has been reported as early as in 1969 by Barbara and Nickel, and Heinz (1969). Due to lab to lab variations and varietal differences the existing protocols for regeneration did not work well in our hand with CO 290 variety of sugarcane which we have selected as our experimental material. Therefore we worked out a more refined protocol for the high frequency plantlet regeneration suitable for our variety CO 290. Nadar et al (1978) claimed the occurance of 'embryoids' in sugarcane callus cultures maintained on a high auxin medium and postulated that a low auxin concentration may be required for advanced stages of embryogenesis. However, these workers failed to show full somatic embryo development. Alloowalia and Maretzki (1983) also reported regeneration of sugarcane plants through somatic embryogenesis. We have successfully induced somatic embryogenesis from long term (10 week old) callus cultures derived from leaf explants and subsequent differentiation of these embryoids into complete plantlets.

Chromosomal mosaicism has been observed by Taskal and Hutchinson (1974), Krishnamurthy and Taskal (1974) and Heinz and Mee (Unpublished data) in the regenerated sugarcane clones. Gross Karyotypic changes, cryptic chromosomal rearrangements, somatic crossing over and sister chromatid exchanges and gene amplification and

diminution were found to be the underlying reasons for the somaclonal variations among plantlets derived from callus cultures of sugarcane (Larkin and Scowcroft, 1983). Further, somaclonal variation has been rightly exploited for developing disease resistant plants of sugarcane to Fiji disease by Krishnamurthy and Tlaskal (1974) and to the eye spot disease by Heinz (1973).

However not much research has been done in the qualitative or quantitative variations in the DNA and the relevance of such variations with respect to the phenotypic or genotypic characters among the regenerated sugarcane plants in vitro.

DNA molecule denatures precisely at characteristic temperature detected by an increase in the absorbance at 260 nm or a decrease in the viscosity of its solution. Each species of DNA has a characteristic denaturation temperature or melting point. Higher the G≡C content higher the melting point when compared to A=T content. Therefore we have adopted this molecular parameter to screen the regenerants with respect to variation in gross sequence changes in DNA.

MATERIAL AND METHODS

Initiation of Callus cultures -

Young leaves of the innermost whorle as well as old leaves were selected as explants for callus initiation. Two types of calli were formed from the foliar explants on MS medium containing 3mg/l 2,4-D, 10% coconut water, 1mg/l Thiamine HCl & 0.8% agar as solidifying agent. One is highly nodular and the other is friable, the base of the young leaves gave rise to nodular callus while the older leaves gave rise to a friable callus. The range of morphogenetic effects was studied in the culture by altering the concentration of 2,4-D and coconut milk. Phenolic exudation from cultures in the medium was prevented by the addition of 50 to 100 mg/l each of ascorbic acid and citric acid in the medium.

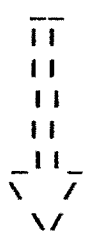
Plant Regeneration via organogenesis -

The callus obtained from the inoculated leaf disks was subcultured at every 2 week interval on the same medium used for callus induction. Callus was incubated in complete dark. Actively growing 4 week old dark grown callus was transferred to the MS medium supplemented with 10% v/v coconut milk, 1 mg/l Thimine HCl and devoid of

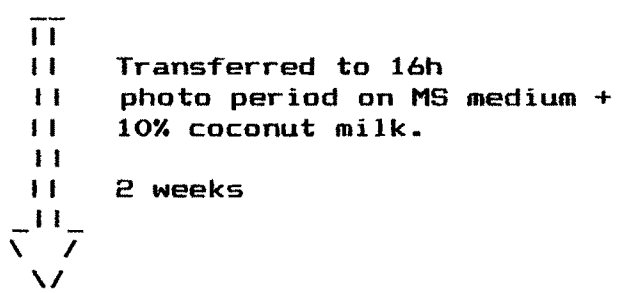
2, 4-D and incubated in 16 hour photoperiod. Well differentiated shoots from callus cultures appeared after fifteen days. They were separated and were placed for rooting either on filter paper bridges in white's basal medium or in the MS medium supplemented with 1 mg/l IBA.

SUGARCANE - REGENERATION PROTOCOL

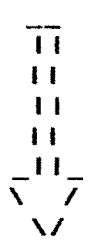
Callus initiation from young leaf explants of sugarcane var. CO 219 on MS + 3 mg/l 2-4,D, 10% v/v coconut milk, 2% sucrose, 0.8% agar. pH 5.8



Callus proliferation



shoot differentiation. Individual shoots transferred to MS basal + 1 mg/l IBA



Profuse root formation

SUGARCANE - SOMATIC EMBRYOGENESIS

Explant - young leaves.
Variety - CO 290

MS basal + 3 mg/l 2,4-D + 10% v/v
coconut water + 3% sucrose (= MSC Medium)
pH 5.8, 0.8% Agar

||
|| 2 Weeks
||
\/

Nodular Callus

||
|| Prolonged culture for 10 weeks
|| on MSC medium in dark.
\/

Induction of Somatic embryogenesis from callus

||
|| Transfer to MS basal +
|| 10% coconut water +
|| 3% sucrose, pH 5.8,
|| 0.8% Agar. (= MSD Medium)
\/

||
|| After 4 weeks
||
\/

Complete plantlets with root & shoot axis

||
|| Plants transferred to
|| Half strength MS basal +
|| 3% sucrose, pH 5.8,
|| 0.8% Agar. (= MSB Medium)
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||
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Plants of 10-15 cm height with well developed roots.

Plant regeneration via embryogenesis -

Prolong subculture of (10 to 12 week) nodular callus on medium containing MS basal supplemented with 3 mg/l 2,4-D, 1 mg/l thiamine HCl and 10% coconut water (MSC medium) in dark triggered somatic embryogenesis. Squash preparations were made from these cultures and stained with safranin. Further differentiation of embryo into complete seedling with root and shoot was achieved when transferred to MS medium supplemented with 10% coconut water and without 2,4-D (MSD medium). Further growth of the plantlet to 10 to 15 cms with well developed root was achieved on medium containing half strength basal MS medium (MSB medium). Complete protocol for somatic embryogenesis is outlined in the Page no 153 .

Plant acclamatization:

Well developed plants of 5-6 inches in length which were obtained via organogenesis as well as embryogenesis were transferred to several sterilized potting mixtures consisting of equal amounts of soil and vermiculate, sand and vermiculate and pure sand. Plants were transferred to the green house where high humidity and low temperatures were maintained initially. Plants were watered frequently, nurtured with half strength Hoagland

solution and sprayed with solution containing 1 g/l Bavastin and 100 mg/l streptocyclin at every fortnight to check the disease occurrence.

Extraction and Estimation of DNA:

Keeping in view the lengthy process of extraction and estimation of DNA and its GC base ratio, we have processed only 10 randomly selected regenerants derived from Callus along with control which constituted plant derived from conventional nodal cuttings. The method described by Cherry (1967) was followed for the extraction of DNA as described below briefly:

A known weight of tissue (500 mg) was homogenized throughly at room temperature in the extraction buffer (1:2 w/v) by adding a pinch of glass powder. 125 ul of 10% SDS was added to this and the homogenization was continued for another minute.

Homogenate was transferred to polypropylene tubes and incubated in water bath at 65°C for 30 minutes.

Tubes were transferred to room temperature and about 350 ul of 8M potassium acetate was added and the tubes were

incubated in the ice for one hour.

|
V

Finally the homogenate was centrifuged at 5000g for 10 minutes at 40°C. Pellet was discarded and supernatant was processed as given below:

|
V

To the supernatant 2 ml chloroform and 5.5 ml phenol were added and the mixture was vortexed to mix throughly. The upper aqueous layer was transferred to another test tube and this step was repeated.

|
V

5 ml of ethanol was added to precipitate the DNA by incubating the mixture on ice for 30 minutes.

|
V

The mixture was centrifuged at 5000g at 0°C for 10 minutes to pellet the precipitated DNA. Supernatent was discarded and pellet was air dried at room temperature. The DNA was resuspended in 300 µl of T.E. buffer.

Quantification of DNA :

Total DNA content was determined using Diphenyl amine (DPA) reaction (Burton 1956). Briefly an aliquote of

DNA was diluted to 2.0 ml with 0.5N $HClO_4$ (0.5N) and mixed with 4.0 ml of DPA reagent. The mixture was incubated overnight (12 hour) in waterbath at 30°C. Absorbance of the coloured solution was measured at 660 nm and compared with the standard curve prepared using Calf thymas DNA (Sigma) to calculate DNA content.

Preparation of DPA reagent :

1.5 gm of purified DPA was dissolved in 100 ml glacial acetic acid. To this 1.5 ml concentrated H_2SO_4 was added and the mixture was stored in dark. At the time of use, 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added for each 20 ml of reagent required.

Preparation of T:E: buffer :

0.5 ml of 2 M Tris HCl (pH 7.4) was mixed with 20 µl of 0.5 M EDTA pH 8 and 99.5 ml of Distilled H_2O. The solution was stored at room temperature.

Preparation of SS phenol :

500 gms phenol, 100 ml of 2M Tris (pH 7.4) and 130 ml of water were mixed and heated to 40°C till the phenol is completely dissolved. This solution was transferred to a separatory funnel. The upper aqueous phase was

discarded. To the lower phase 100 ml of 2M Tris (pH 7.4), 2 ml of metacresol, 1 ml of ß-mercaptoethanol and 500 mg of 8 hydroxy-qunoline were added. This solution was stored in a brown bottle at room temperature.

Measurement of Tm value of DNA :

A small aliquote of sample DNA extracted from 500 mg of tissue was dissolved in 2 ml of T.E. buffer and was placed in a temperature controlled cuvette of a UV-VIS spectrophotometer (Shimazdu, Japan). The DNA was subjected to thermal denaturation or strand separation by controlled heating in the cuvette. At every minute 2.5°C temperature was raised. The optical density of the solution was continuously recorded at 260 nm. When no further increase in absorbance occurs the denaturation is considered to be complete. The Tm curve was prepared by plotting temperature against the absorbance. (FIG 14). The temperature corresponding to half the increase in the relative absorbance is designated as Tm. The % mole GC content of each plant was calculated by using formula:

$$\% \text{ mole GC} = (Tm - 69.5)\ 2.44$$

RESULTS

CALLUS INITIATION AND PROLIFERATION:

Callus was developed from cut ends of leaf base within two weeks of inoculation. Accumulation of polypohenols in callus was checked by its rapid transfer into fresh medium, sometimes at every two days interval. But this laborious procedure was substituted by addition of 50 mg/l each of ascorbic acid and citric acid in the medium which successfully prevented phenolic accumulation in callus. Growth of the callus required its subculturing every month. Friable callus showed great potential of organogenesis when compared to nodular callus.

PLANT DIFFERENTIATION VIA ORGANOGENESIS:

Four week old friable dark grown callus when transferred to MS medium devoid of 2,4-D differentiated into shoots after 15 days (Fig.12.B). Callus incubated under continuous illumination also differentiated into shoots but these shoots were not very vigorous.

Root formation occured profusely from these shoots when transferred on a medium containing 1 mg/l IBA.

PLANT DIFFERENTIATION VIA EMBRYOGENESIS:

FIG.12. Sugarcane orgonogenesis

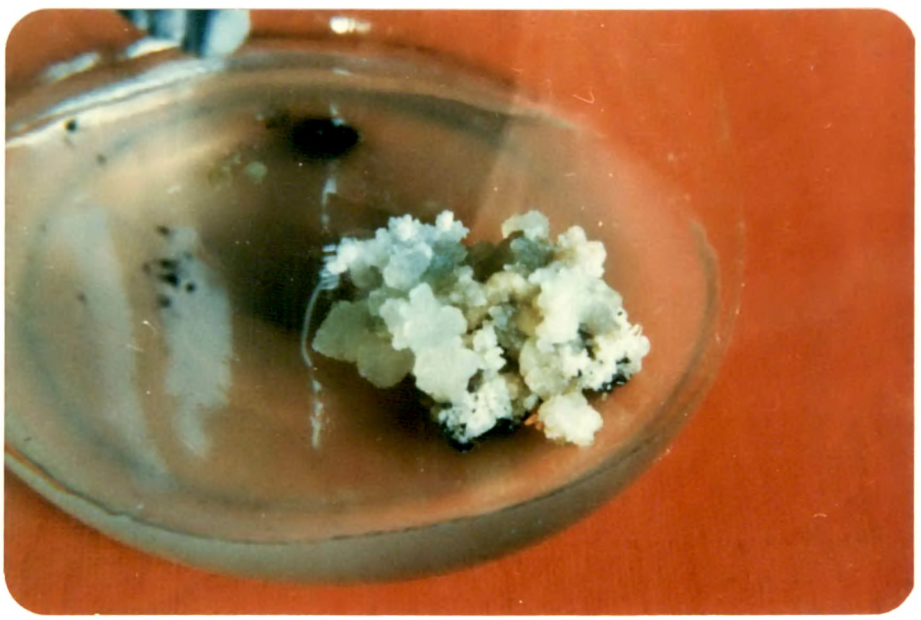
A. Leaf originated friable callus on MS + 3mg/l 2,4-D + 10% v/v coconut milk and 2% sucrose.

B. Shoot differentiation from callus on MS basal with 10% coconut milk + 1 mg/l Thimine HCl + 2% sucrose.

C. Complete plants with well developed roots on MS basal + 1 mg/l IBA.

D. Fully grown plant in acclamatization.

* C and D are contd on next page



A

B

C

D

FIG.13. Sugarcane somatic embryogenesis

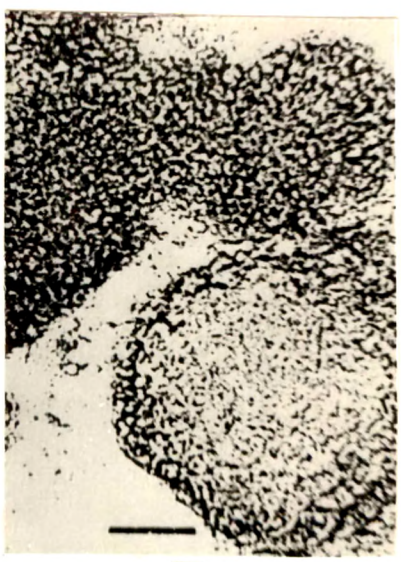
A. Nodular embryogenic callus on MSC medium

B. Globular embryo

C. Advanced stage of somatic embryo on MSD medium

D. Complete plant formation on half strength MS basal with 3% sucrose.

A



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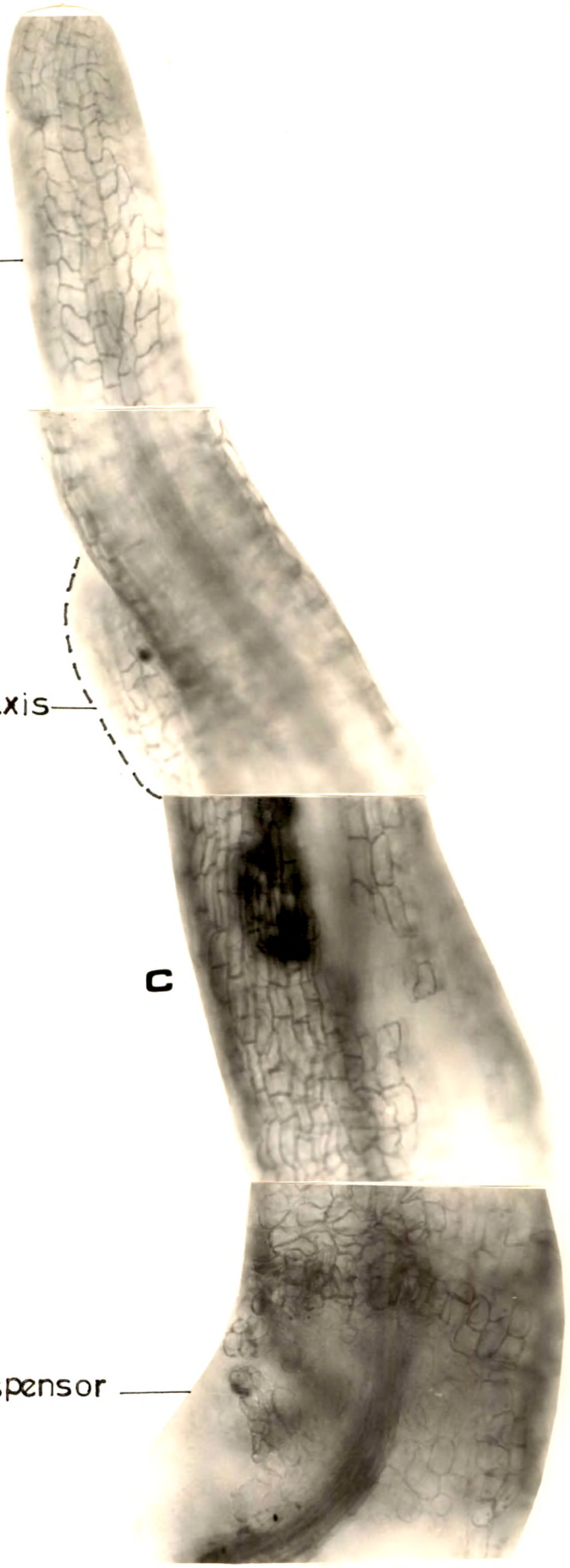

Somatic embryogenesis was obtained from the 10 to 12 week old dark grown nodular callus on MSC medium. (FIG.13.A) Early stages of embryogenesis starting from globular to elongated embryo were observed. (FIG.13.B). Globular embryoids are with typical epidermal layer formed by shield like cells, which completely surrounded the dense meristematic cells. In later stages of embryogenesis differtiation of suspensor, scutellum and root/shoot axis was observed. (FIG.13.C) Further growth of embryo into seedling with well developed root and shoot was achieved on four weeks on MSD medium(FIG.13.D).

PLANT ACCLAMATIZATION:

Plant establishment and survival as much as 80% was observed in potting mixtures consisting of sand and vermiculate (50:50) than in the other combinations of pure sand and soil and vermiculate (50:50). Well grown plants were repotted into polythene bags containing pure garden soil (FIG.12.D).

QUANTITATIVE ANALYSIS OF REGENERANTS FOR DNA CONTENT:

There was a great variation in DNA content from plant to plant in the regenerants. (Table 19). Such variation was not seen in the control non tissue culture plants. In the three non tissue cultured control (plants of same variety from

which the cultures were initiated), the average DNA content was 116 µg g^{-1} fresh weight. This value is comparable with the reported values for other angiosperms. For example the cabbage flowrets containing 50 µg g^{-1} fresh weight and the pea seedling was reported to be 300 µg g^{-1} fresh weight (Harbone. J.B., 1976). Similarly in the dormant epiphyllous buds of Kalanchoe it was found to be 188 µg g^{-1} fresh weight (Jasrai, 1987). In the regenerated plant No. 1, the DNA content was found to be the same as that in control i.e. 116 µg g^{-1} fresh weight. Plant No. 2, 3 and 10 showed comparatively less amount 115 µg g^{-1} fresh weight of DNA than control. The remaining regenerants (Plant No. 4,5,6,7,8 & 9 displayed slightly higher amounts of DNA ranging from 120 - 124 ug g^{-1} fresh weight.

The Tm value of the DNA of the control plant was 85°C. Regenerated plant No. 1,2,3 & 10 had same Tm value as that of control i.e. 85°C. The Tm value of plant no. 4,6,7 & 9 was 86.5°C and the Tm value of plant no. 5 & 8 was found to be 87.5°C. Similarly the % mole GC content of control as well as that of plant no 1,2,3 & 10 was 38.308, whereas the % mole GC content of remaining regenerants 4,5,6,7,8 & 9 was in the range of 41.968 to 44.408.

TABLE NO. 19

DNA AND GC ANALYSIS DATA OF REGENERANTS

Sl.No.	Regenerant No.	Total DNA content $\mu g/g^{-1}$/fresh wt.	Tm in °C	GC content % mole
1.	Control (field grown plants)	116	85	38.308
2.	Plant no. 1	116	85	38.308
3.	Plant no. 2	115	85	38.308
4.	Plant no. 3	115.5	85	38.308
5.	Plant no. 4	120	86.5	41.968
6.	Plant no. 5	123.5	87.5	44.408
7.	Plant no. 6	119	86	40.748
8.	Plant no. 7	120	86	40.748
9.	Plant no. 8	124	87.5	44.408
10.	Plant no. 9	120	86.5	41.968
11.	Plant no. 10	115	85	38.308

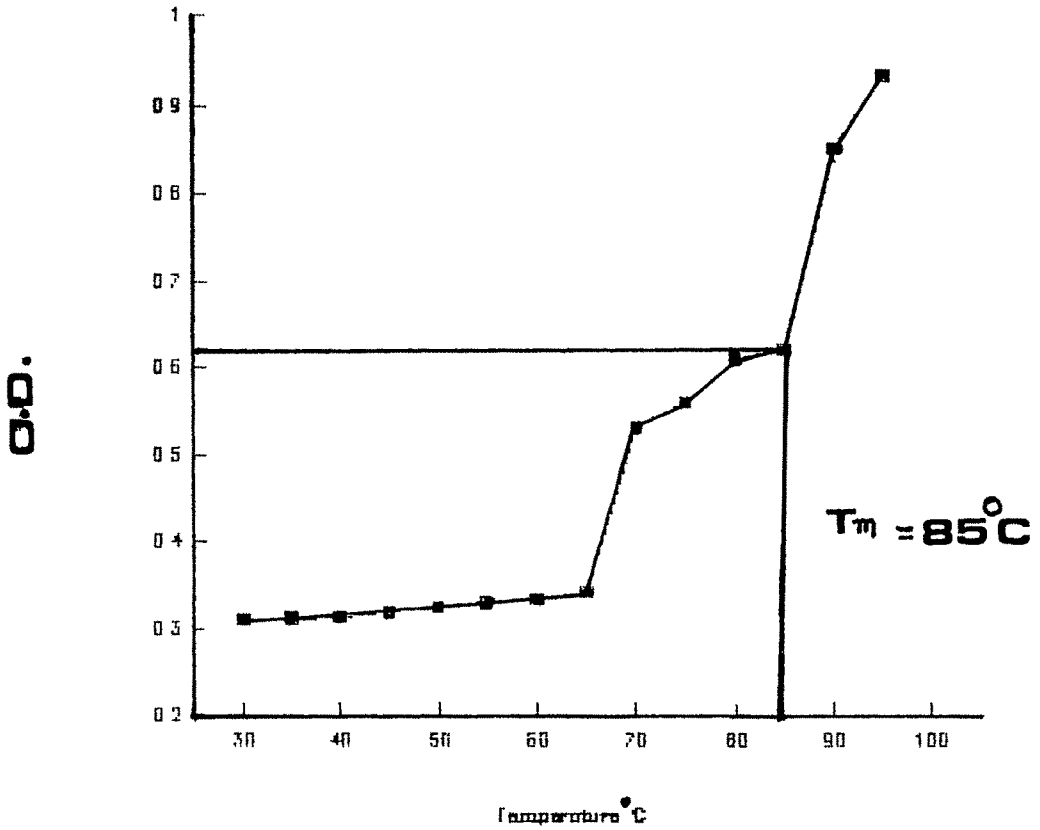

FIG.14. T_m - GRAPH

DISCUSSION

Emergence of nodular callus from the young leaves and friable callus from the old leaves suggests some relationship between the totipetancy of leaf cells on one hand and the leaf age on the other hand. Of these friable callus possessed the greater differentiation potential when compared to the nodular callus. Shoot differentiation occured when dark grown callus transferred to a medium without 2,4-D and incubated in light. Callus when grown in dark never showed any degree of differentiation. Callus continuously grown in light and in the presence of 2,4-D also differentiated into shoots. But shoots derived through this method were not healthy and further these plants did not survive during acclamatization. This indicates that omission of 2,4-D and initial incubation of callus in the dark are necessary for healthy shoot differentiation. Rooting of differentiated shoots was described to be difficult in the earlier reports of Heinz and Mee (1969). Of the two methods described shoots transferred to the medium supplemented with IBA proved to be highly satisfactory. Thus we have developed a refined protocol (Page 152) for the high frequency of plantlet regeneration.

Somatic embryogenesis was earlier reported in sugarcane by Alloowalia and Maretzki (1983). In their protocol, somatic embryogenesis was obtained in 10 week old leaf originated callus on MS medium supplemented with 3 mg/l, 2-4,D and 10% coconut water. Further no mention is made about the nature of the embryogenic callus whether it is friable or nodular and whether it is incubated in light or dark. In our case somatic embryogenesis was induced in the 10 week old nodular dark grown callus on MS medium containing 3 mg/l, 2-4,D and 10% coconut water. The somatic embryoids further differnetiated into complete plant lets upon subculture to MS basal supplemented with 10% coconut water without 2-4,D. Further growth of the plantlet to 10 to 15 cm with well developed root was achieved on medium containing half strength basal MS medium. Thus the protocol described for somatic embryogenesis is complete.

Regenerated plants when screened for DNA and Tm value displayed slight but detectable variations than that of mother plants. Heinz and Mee (1971) studied the regenerated plant populations derived from callus cultures of sugarcane var. H37-1933 and H50-7209. They observed variations in the morphology, chromosome number. Of the 37 plants of H50-7209, all but one had cell to cell variation in chromosome number. Heinz and Mee (1971) described the chromosomal variation due to asynchronous division of multinucleate cells. In all

the above cases variation was well documented at cellular level as well as at plant level. Further these variations could be due to the prolonged exposure of cells to various chemical and growth harmones in the medium. In our case plants were regenerated from the callus of only 3-4 subculture old. This could be the reason for the occurrence of only slight variations in terms of DNA content among the regenerants. The difference in the total DNA content was found to be comparatively more amongst the regenerants from the parent clone than that of the value of difference in the Tm of DNA (Table). The Tm values of plant No. 2,3 and 10 were same as that of control i.e. 85°C and the % mole GC content were 38.308 respectively, but the DNA content was slightly less than that of control to the range of 1 - 0.5 ug/g^{-1} fresh weight. Though there was change in the total DNA content in all these plants from control there was no change in the denaturation time of DNA. The reason could be that the change in DNA content was small so that it did not reflect in terms of Tm value to a detectable level. Where as plant No. 5 and 8 have shown significant increase in DNA content in the range of 7.5 to 8.0 ug g^{-1} fresh weight to that of control along with the higher melting temperature (Tm) to 2.5°C than that of control. Presently we don0t know the significance of altered DNA content as well as altered Tm value in terms of phenotypic expression in these regenerants.