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TISSUE AND CELL CULTURE AS AIDS TO SUGARCANE BREEDING. I. CREATION OF GENETIC VARIATION THROUGH CALLUS CULTURE

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INDEX WORDS

Saccharum species hybrid, sugarcane, tissue culture, induced differentiation, callus-derivative, morphological variation, chromosome mosaicism.

SUMMARY

Four-hundred and seventeen plants from 8 sugarcane varieties were selected among the 4600 plants that had been generated from calluses of shoot-apices, rolled young leaves and young inflorescences of 58 varieties. They were planted in the field (Field Test-II stage) in December, 1971 and examined for morphology, sugar content and chromosome number in the summer of 1972 and thereafter. The callus derivatives of the 8 varieties differed from their donors in the frequency of morphological changes: F146 (1.8%), N:Co310 (2.4%), F161 (4.8%), F164 (6.7%), F162 (7.4%), 56–2080 (15.8%), F170 (27.6%), and F156 (34.0%). Auricle length showed the greatest frequency of difference (8.6%); dewlap shape ranked next (6.5%); followed by hair group (6.2%); attitude of top leaves (1.9%). The sucrose content of callus derivative was increased over their donors by 2% to 12%. Chromosome number (2n) varied from 86 to 126 in the F156 derivatives in contrast with the donor's 114 whereas those of F164 varied from 88 to 108 as compared with the donor's 108. The derivatives of F146 generally centred around the original chromosome number of 110 and it is considered to be the most genetically stable cultivar. The cause of the occurrence of chromosome mosaicism in sugarcane is discussed. There was no apparent correlation between morphological modifications and changes in chromosome number.

INTRODUCTION

The present study is one of a series of experiments dealing with the evaluation of callus-derived plants with particular emphasis on the detection of variation in morphology, sugar content and karyology. It is investigated whether there is any relationship between morphological alterations and changes in chromosome number.

Variation in chromosome number within plants obtained through tissue-culture has been reported by MURASHIGE & NAKANO (1966), MELCHERS et al. (1969), SACRISTAN & MELCHERS (1969), TLASKAL et al. (1970), and NIIZEKI & OONO (1971). HEINZ & MEE (1971) have also shown that there is appreciable genetic variation amongst subclones derived from sugarcane callus tissue. KRISHNAMURTHI & TLASKAL (1974) recently reported on some shifts in chromosome number in a sugarcane callus derivative whereby they had obtained a disease resistant sub-clone. But, none of them

ever analyzed the sugar content of the regenerated plants which is the most important character in a commercial sugarcane variety.

MATERIAL AND METHODS

Initiation of callus from subapical meristems, rolled young leaves and young inflorescences of sugarcane was achieved on a modified MURASHIGE & SKOOG medium (1962) as reported in a previous paper (LIU, 1971). Promotion of shoot differentiation in calluses and procedures for rearing the regenerated plants were outlined by LIU et al. (1972).

Some 4600 single plants were regenerated from calluses of 58 sugarcance varieties including F (Formosa) varieties, introduced strains (e.g., N: Co310) and promising Taiwan-bred lines (e.g., 56–2080). They were individually planted in the field. This compares to the Field Test (FT)-I stage of a cross-breeding program. A total of 417 plants from eight varieties among the above mentioned regenerated plants were selected at maturity (i.e., from December to February) for better performance than their donors. These 417 plants were vegetatively propagated in December, 1971 with 3 two-node setts per meter in 6-meter rows at a distance of 1.25 m between rows. This compares to the FT-II stage of a conventional cross-breeding program.

Attitude of top leaves, auricle length, dewlap shape and hair group as described by ARTSCHWAGER (1939) were carefully examined in comparison with their donors. A chi-square test for independence of the ratio of morphological alteration to non-alteration on clonal genetic constitution, with a 8×2 contingency table and 7 degrees of freedom, was run in accordance with STEEL & TORRIE (1960).

To illustrate the differences in auricle length, several callus derivatives differentiating from F156, F164 and 56–2080 were selected for detailed investigation. Twenty tillers were randomly chosen from the plants in each row and their auricle lengths located at the joint of leaf blade and sheath were measured for the five top leaves. To compare the donor with its derivatives, d' (DUNNETT's standard deviation of the difference) was calculated (DUNNETT, 1955). In the same procedure, the degrees of curvature of the leaf blade as measured by the tangential angle at the point where the blade starts to curve (e.g., 180° denotes straight, 155° the most bend one) were employed to distinguish the angle of top leaves between the paired materials (donors vs. their derivatives). In the same sense, the differences were tested by DUNNETT's d'.

In order to isolate a valuable callus derivative expected to be improved in a single defective character, all the materials including those which are derived from calluses and those which are sexually produced were routinely analyzed for available sugar content by a small mill method (carried out by the Chemical Analysis Laboratory, Taiwan Sugar Research Institute) each year. Some callus derivatives were analyzed in consecutive months or years beginning from FT-II stage and thereafter in order to generalize reliable data.

Callus derivatives were randomly collected from F146, F156 and F164 populations for karyological examination. To count the chromosomes in pollen mother cells young spikelets were fixed in NEWCOMER's solution (NEWCOMER, 1953) and smeared in 45% iron aceto-carmine. Somatic cells were examined by the leaf squash method described by PRICE (1962). Five to ten cells per plant were examined.

RESULTS AND DISCUSSION

Morphological variation in callus-derived plants. The number of callus derivatives differing in morphological characters from their donors is listed in Table 1. The F156 population had the highest percentage of plants (34.0%) differing morphologically from its donor; the F170 ranked next (27.6%), followed by the 56–2080 (15.8%). The F146 population exhibited the lowest frequency of visible differences (1.8%). A highly significant ($p<0.01$) contingency chi-square, 58.7, was obatined (Table 1). Accordingly, it is concluded that the ratio of plants with morphological changes to those without changes varied, by more than chance, from clone to clone depending upon the clonal genetic make-up.

The mean auricle lengths for F156 and its derivatives, 70–6128, 70–6406 and 70–6075 were 4.39, 1.40, 2.29 and 1.80 cm, respectively (Fig. 1). Individual comparisons between the donor and its derivatives were tested by DUNNETT's procedure and each turned out to be significant at the $p<0.05$ level. DUNNETT's standard deviation of the difference (d') was 0.58 cm. The means for F164 and its derivative, 70–8133, were 0.38 and 0.88 cm, respectively (Fig. 1). The former was significantly shorter than the latter if DUNNETT's d' (0.24 cm) was used as a criterion. In total, 8.6% of the derivatives showed alterations in auricle length, which was the highest frequency for any of morphological characters studied.

The attitude of the top leaves of the original F146 is mostly straight (178°). However, its derivative, 70–6111, had leaves hanging at an angle of 162° (Fig. 2). The difference (16°) was significant at the $p<0.05$ level. The mean leaf curvatures of

Table 1. Number of callus derivatives[1] differing in morphological characters from their donor clones.

Donor clone	Number of derivatives surveyed	Number of callus derivatives showing differences in									
		top leaf attitude	auricle length	dewlap shape	bud shape	stem color	hair group 55	hair group 57	hair group total	total derivatives number[2]	total derivatives %
F146	57	1	0	0	0	0	0	0	0	1	1.8
F156	50	2	3	0	1	2	5	17	22	17	34.0
F161	21	0	1	1	0	0	0	0	0	1	4.8
F162	27	0	2	0	2	0	0	0	0	2	7.4
F164	15	0	1	0	0	0	0	1	1	1	6.7
F170	105	1	27	24	1	0	0	0	0	29	27.6
56-2080	19	2	1	0	0	2	0	2	2	3	15.8
N:Co310	123	2	1	2	2	0	0	1	1	3	2.4
Total	417	8	36	27	6	4	5	21	26	57	
Pooled (%)		1.9	8.6	6.5	1.4	1.0			6.2		13.7

[1] Callus derivatives in 6-meter rows in the FT-II stage.

[2] Callus derivatives with more than one difference in morphological characters were counted only once. Contingency chi-square with 7 degrees of freedom was 58.7 ($p<0.01$), indicating that the ratio of morphological alterations to non-alterations varied by more than chance, and is not independent of the original clonal source.

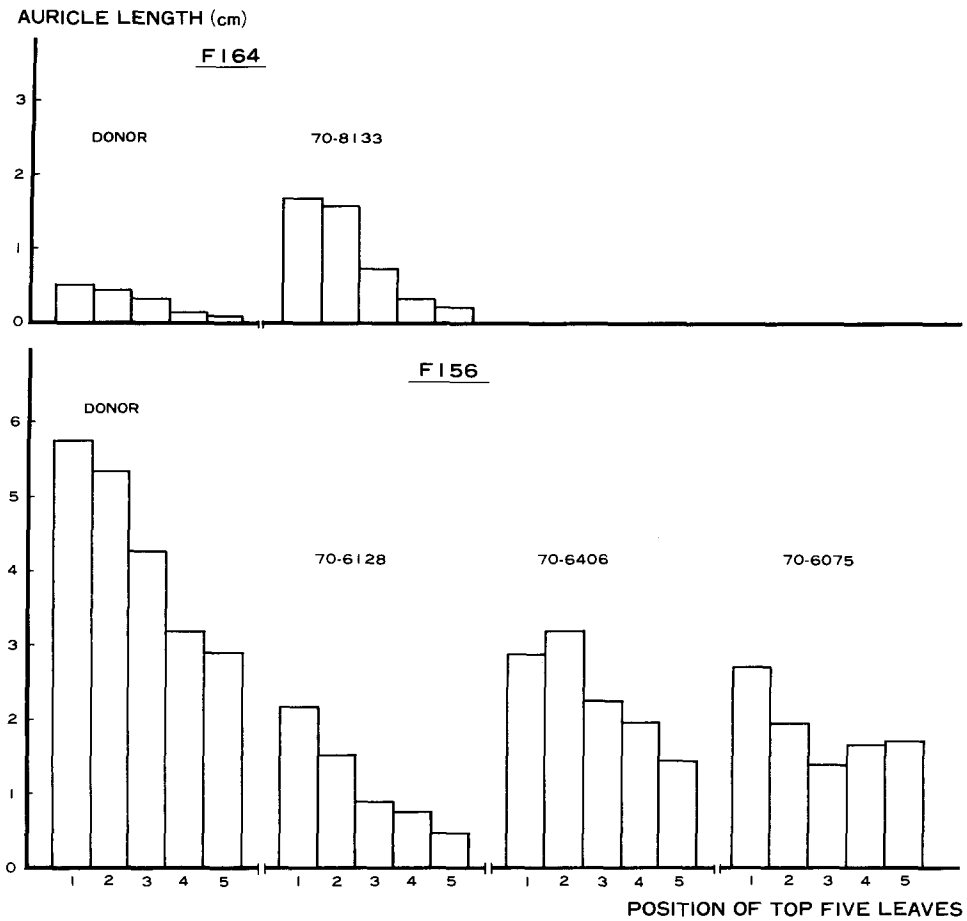

Fig. 1. Histogram of auricle length of the five top leaves of the F156 and F164 derivatives which differed markedly from their donors (No.1 denotes the first unrolled leaf, No. 2 the second, etc.).

F156 and its derivatives, 70–6072, 70–6128 were 177°, 178°, and 167°, respectively (Fig. 2). The donor was not significantly different from 70–6072, but was significantly different from 70–6128 (d' = 2.27°). The mean leaf curvatures of 56–2080 and its derivatives were 175°, 163° and 166°, respectively. The differences between the original and 70–6477, and between the original and 70–6482 were significant at the $p<0.05$ level with d' = 3.52°. The two derivatives were similar in leaf attitude. In total, 1.9% of the plants varied from the donor with respect to attitude of top leaves.

Dewlap shape is also a good indicator for detecting differences between the related paired populations. It ranked next to auricle length in showing discrimination between the donor and the callus derivatives (6.5%).

The presence or absence of hair groups, which characterize certain varieties, also reveals differences between the original and its derivatives. It accounted for 6.2% of the derivatives showing changes (Table 1).

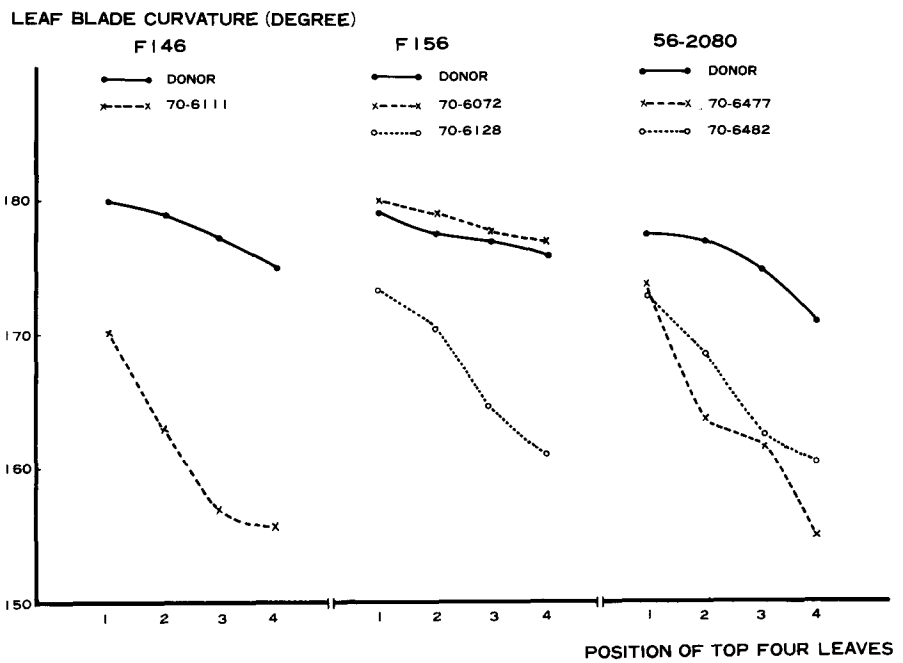

Fig. 2. The attitude of the four top leaves of the F146, F156 and 56-2080 derivatives which manifested the most visible changes as compared with their donors (No. 1 denotes the first unrolled leaf, No. 2 the second, etc.).

Variation in sugar content of callus-derived plants. Table 2 presents the variations in the available sucrose content of several callus derivatives. The analyses of sugar content were repeated for consecutive months in one or two crop years (1973/1974, 1974/1975). It is evident that the average sugar content of all callus derivatives but one was increased over their donors by 2% to 12%. Such increment of sugar content will contribute greatly to the sugar industry if calculated on a large-scale basis.

Karyological variation in callus-derived plants. Chromosome counts, prepared by either the pollen mother cell smear method or the leaf squash method, were made for the 22 plants derived from F146, F156 and F164 populations. The results are summarized in Table 3. Some examples of chromosome variation are shown in Fig. 3–12.

The chromosome numbers of the leaf cells of the F156 population varied from 86 to 126. Such a large variation in chromosome complements could well be reflected in the high frequency of morphological changes (Table 1). One striking plant, F156–70–6128, with a chromosome number of about 2n = 101 showed not only a change in auricle length but also in attitude of top leaves as compared with the donor which has a somatic number of 114 (Table 3, Fig. 1–3).

Two cells from the same growing point of F156–70–6069 had chromosome num-

Table 2. Variations in available sucrose content of callus derivatives as compared with their donors.

Derivative	Available sucrose (%)					
	1973/1974[1]			1974/1975[1]		
	donor	derivative	%	donor	derivative	%
F156[2]-70[3]-6069	13.31	13.66	103	13.76	12.70	92
F156-70-6130	13.31	14.11	106	13.76	13.98	102
H37-1933-71-6127	13.47	14.06	104	13.47	15.10	112
Q85-71-6122	14.24	14.56	102	13.52	14.23	105
Q84-71-6125	13.68	14.47	106	–	–	–
F157-71-6124	–	–	–	12.62	13.14	104
F164-70-6132	–	–	–	13.38	13.62	102
F164-70-6136	–	–	–	13.38	13.75	103

[1] Average of 3-5 month-sucroses analyzed in the period of October to February.
[2] The donor.
[3] The year in which the callus cerivatives were transplanted in the field from a sterilized medium.

Table 3. Chromosome number of several plants derived from callus of sugarcane varieties F146, F156 and F164.

Genetic material	Number of chromosomes (2n = ca.)	Genetic material	Number of chromosomes (2n = ca.)
F146 (Donor)	110[1]	F156-70-6131	95–114
F146-70-6106	110[1]	70-6134	92- 98
70-6052	104–112[1]	70-6139	88–102
70-6057	108–110[1]	F164 (Donor)	108
70-6367	109–110[1]	F164 70-8501	102
70-6369	112[1]	70-8507	106
70-6372	110–180[1]	70-8527	94–107
70-6383	106-108[1]	70-8532	104–108
70-6400	116–118[1]	70-8541	89–108
F156 (Donor)	114	70-8544	91–104
F156-70-6069	113–126	70-8549	90
70-6080	86–93	70-8562	88–91
70-6128	101–115		

[1]Counts from pollen mother cells; the others are from leaf squash.

bers of 113 and 122, respectively (Fig. 4–5). The occurrence of different chromosome numbers in the same plant tissue has been frequently reported. TLASKAL et al. (1970) have demonstrated that cells in a single microscopical slide prepared from sugarcane young leaf tissue had chromosomes of 98, 110, 121, 126, 135 and 143. SPECKMANN & VAN DIJK (1972) also reported that a considerable variation in chromosome number occurred in individual plants of *Poa pratensis*.

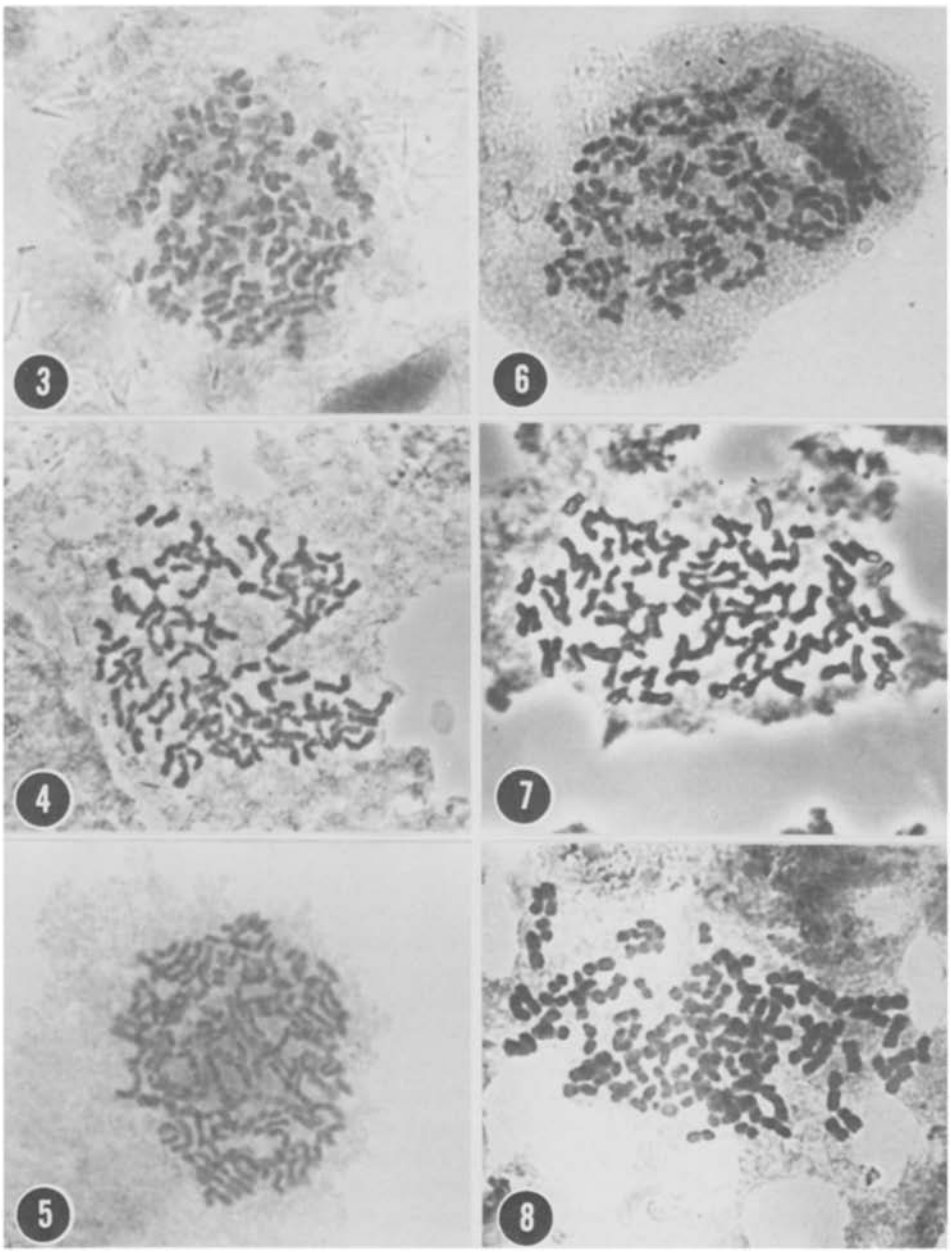

Fig. 3–5. Chromosome at metaphase in leaf cells of the F156 derivatives. Fig. 3. Mitotic metaphase with 2n = 101 (ca.) in a cell of 70-6128 which showed morphological changes. Magnification × 1350. Fig. 4. Metaphase with 2n = 113 (ca.) in a cell of 70-6069. Magnification × 1350. Fig. 5. Another cell of 70-6069 with a chromosome number 2n = 122 (ca.). Magnification × 1350.
Fig. 6–8. Mitotic metaphases in leaf cells of the F164 derivatives showing variations in 2n number. All photographs for chromosome count were taken with bright illumination except Fig. 4 and 7 which were taken with a Zeiss phase-contrast microscope.

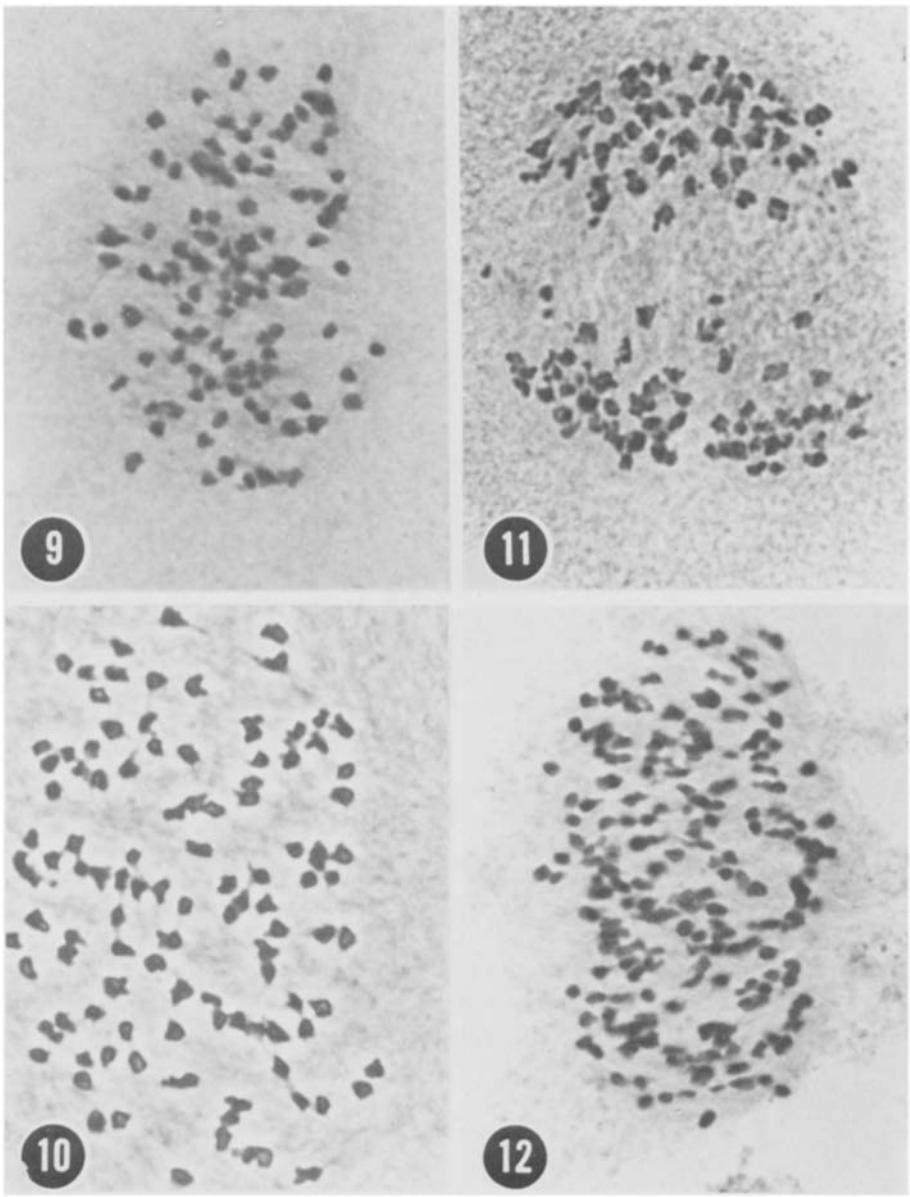

Fig. 6. 70-8501 having 102 (ca.). Magnification × 1350. Fig. 7. 70-8507 having 106 (ca.). Magnification × 1350. Fig. 8. 70-8541 having 108 (ca.). Magnification × 1350.
Fig. 9–12. Microsporocyte chromosome of the F146 derivatives prepared by the pollen mother cell smear method. Fig. 9. Late metaphase I with $6_{II} + 94_{I}$, the six bivalents can be seen in a cell of 70-6383. Magnification × 1280. Fig. 10. Early anaphase I in a cell of 70-6106, 2n = 110. Magnification × 1280. Fig. 11. Anaphase I in a cell of 70-6400 showing homologous chromosomal separations, some lagging chromosome can be seen. Magnification × 1280. Fig. 12. Late metaphase I of 70-6372 with an unexpected chromosome number of 2n = 180 (ca.), some ot them were overlapping. Magnification × 1280.

Somatic chromosome numbers of the F164 derivatives with a range of 88–108 varied a little less than those of the F156 derivatives which had a range of 86–126 (Table 3). As in the F156 population, most of the F164 derivatives tended to be lower in chromosome number than the donor (2n = 108). The reason will be explained later in this paper. The lower variation in chromosome number in the F164 population also agreed well with a lower percentage of morphological changes.

Chromosome numbers from pollen mother cells of the F146 derivatives are designated by 2n numbers since most of the bivalents had disjoined at the stages illustrated in Fig. 9–12. The chromosome numbers of the F146 derivatives generally centred around the original number, 2n = 110, and were much more stable than either of the other two groups. The chromosomal stability in the F146 population is in line with its morphological characteristics in which only minimal differences were detected (1.8%) (Table 1).

In looking for hypotheses to elucidate the occurrence of chromosomal mosaicism in higher plants, the following possibilities must be considered:

1 Complex ancestry. HEINZ & MEE (1971) attributed the chromosome instability of H50–7209 to its complex genetic nature since it had been derived from germ plasm of at least three species of *Saccharum*. Others (HEGWOOD & HOUGH, 1958; NIELSEN & NATH, 1961) have supported this idea. However, this explanation may not be applied in the present case. An exemination of the ancestry of F146 and F156 shows that the former is more complex in genetic composition than the latter since F146 is the product of N:Co310 × P.T.43–52, whereas F156 is the progeny of F141 × C.P. 34–79. F141 and C.P. 34–79 derived more of their germ plasm from noble cane (*S. officinarum*) than did N:Co310 which contains mostly the germ plasm of wild cane, *S. spontaneum* (India). However, the F156 population varied more in chromosome number than that of F146.

In addition to the differences in variation of chromosome number of these two populations which might be due to the counts from two distinct tissues (e.g., leaf tissues versus pollen mother cells, the former is usually more variable than the latter), the F156 population had higher frequency of morphological variation than did the F146 group. The situation of these two populations thereby provided an another evidence against the 'complex ancestry' hypothesis since F146 was the most genetically stable clone of the groups studied (Table 1) but contains more complex germ plasm than does F156.

2 Stemline theory. According to MAKINO (1957), certain animal or plant tissue contains several cell populations, each with different chromosome number. Some of these different cell populations could be separated by way of callus formation in vitro whereby complete plants are regenerated. Consequently, such derived plants would have each a different chromosome number. This theory could best explain the existence of callus derivatives genetically different from the original clone. KRISHNAMURTHI & TLASKAL (1974) also agreed with this theory.

3 Fractions exist in chromosome complement. KRISHNAMURTHI & TLASKAL (1974) have suggested that the chromosome complement in a cell is not a single entity and that 2 or more fractions may coexist. They have shown a plate with 2 fractions of chromosome complement dividing asynchronously. In the same sense, it is also possible that 2 or more fractions may divide synchronously and this will be doubling

or tripling the chromosome number resulting from the divisions of all fractions in a cell. This mechanism could best elucidate the case of F146–70–6372 in which an unusual number 2n = 180 (Fig. 12) was observed.

4 Multipolar meiosis or mitosis. This mechanism often causes the formation of microcells which usually have a lower chromosome number than the original (TAI, 1970). In this study, there were several derivatives showing a considerably lower chromosome number than their donors, e.g., F156–70–6080 (Table 3).

5 Desynapsis during meiosis. This mechanism was operating as shown in the experimental data of JAGATHESAN & SREENIVASAN (1971) and NAIR (1972). Fig. 11 shows an anaphase I cell from the F146–60–6400 sample in which it was apparent that some chromosomes remained lagging on the side.

The examples given above suggest that all of these mechanisms may be operating in the original materials and their derivatives. The relative importance of each is difficult to determine.

Although the real cause of inducing the occurrence of chromosome mosaicism in sugarcane has not been established, this study demonstrates that tissue culture techniques, as we routinely use them for improvement of a single defective character, are able to produce potentially promising new sugarcane varieties.

Of the 22 plants, which were randomly selected for karyological study, all but one plant (F156–70–6128) showed no apparent correlation between morphological changes and variations in the chromosomal complement. This lack of correlation was also observed by SPECKMANN & VAN DIJK (1972), and HEINZ & MEE (1971). In plants derived from tissue culture, major changes in chromosome complement often do not appear to be accompanied by corresponding changes in morphology. Nevertheless, the authors believe that genetic shifts are definitely responsible for the modification of morphology. This idea can be verified by the fact that the frequency of morphological changes depends upon varietal genotypes as illustrated by the data presented in Table 1. The gain or loss in chromosomes sometimes may not be sufficient to cause a large change in morphological characters, and it is even possible that changes can occur that are not visibly expressed.

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