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METHODOLOGY

EVALUATION OF CHROMOSOME NUMBER STABILITY IN TWO SUGARCANE VARIETIES*

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ABSTRACT

During an investigation of the stability of chromosome numbers of plants regenerated *in vitro* from sugarcane varieties, we developed a technique to obtain clearly intact somatic metaphase cells, showing details of the morphology of the chromosomes and with a low degree of chromosome overlapping. Some root tip pretreatments were tested and the combination of 8-hydroxyquinoline plus cycloheximide and α-bromonaphthalene with cycloheximide produced the best results, respectively for the varieties NA56-79 (2n=114) and Co419 (2n=113). A criterion to evaluate the accuracy of chromosome counts was adopted and the results showed that these varieties have chromosome stability.

INTRODUCTION

Modern sugarcane varieties are complex polyploids sysnthezised from at least three or four *Saccharum* species. The cytological study of interspecific hybrids *S. officinarum* x *S. spontaneum* carried out by Bremer (1961a,b) is a good illustration of the complexity of sugarcane cultivars. He observed an increase in chromosome numbers in these hybrids, which had the sum of maternal (*S. officinarum*, 2n=80) somatic chromosome number plus the paternal (*S. spontaneum*, 2n=40 to 128) gametic number. Therefore, most of the sugarcane cultivars have more than 100 chromosomes. Furthermore, chromosomal mosaicism has been detected in some cultivars and somaclones (see Sreenivasan *et al.*, 1987; Heinz, 1991).

Chromosome instability in sugarcane clones makes necessary special precautions for the correct interpretation of chromosome counts and for the detection of mosaicism. Tlaskal and Hutchinson (1973) pointed out three basic sources of variability in sugarcane chromosome counts: a) cells may not be intact and consequently not have a full complement; b) interpretation errors mainly due to chromosome overlapping; c) occurrence of chromosome mosaics.

Several authors have described techniques which enabled them to count chromosomes of sugarcane varieties with accuracy (Price, 1962; Heinz *et al.*, 1969; Tlaskal and Hutchinson, 1974). The fact that each laboratory has developed its own procedures to evaluate sugarcane chromosome numbers has been emphasized by Tlaskal and Hutchinson (1974). During an investigation on the occurrence of chromosome instability in plants regenerated *in vitro*, we developed a root tip squash technique and a procedure to evaluate the accuracy of chromosome counts in sugarcane varieties, based on a method described by Tlaskal and Hutchinson (1974), in which they employed a pretreatment with a combination of 8-hydroxyquinoline and cycloheximide, an antibiotic that inhibits protein synthesis and contracts chromosomes both at prophase and metaphase (Wilson, 1950). The

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procedure adopted for the evaluation of the chromosome numbers of two sugarcane varieties is described in this paper.

MATERIAL AND METHODS

NA56-79, an Argentinean sugarcane variety that has been cultivated in Brazil and has proved to be a very stable clone (Nunes Jr., 1987) and its progenitor, the Indian variety Co419 (Dutt and Rao, 1956) were used. Single-budded cane cuttings were germinated in plastic boxes containing wet *Sphagnum* moss at 30°C. Roots 1-3 cm long were excised and pretreated with "c-mitotic" agents (colchicine, 8-hydroxyquinoline or α-bromonaphthalene) alone or combined with cycloheximide (Actidione), with or without additional DMSO (dimethyl sulfoxide), at room temperature (28-30°C) (Table I). The criteria adopted to evaluate these treatments were number of roots analysed

Table I - Evaluation of pretreatments of root meristems of varieties NA56-79 and Co419.

Pretreatment	Duration (hours)	No. of roots analysed	No. of metaphases selected
NA56-79			
A) Isolated			
0.03% 8-hydroxyquinoline + DMSO[1]	3	10	-
0.1% colchicine + DMSO[1]	3	10	-
0.1% colchicine + DMSO[1]	4	16	-
1.0% α-bromonaphthalene	2	11	1
1.0% α-bromonaphthalene	3	16	-
2.0% α-bromonaphthalene	2	8	-
0.007% cycloheximide	3	5	-
B) Combined[2]			
Cycloheximide + colchicine[3]	3	6	-
Cycloheximide + 8-hydroxyquinoline	4	32	8
Cycloheximide + 8-hydroxyquinoline	5	44	3
Cycloheximide + 8-hydroxyquinoline[4]	5	45	20
Cycloheximide + 8-hydroxyquinoline[4]	5.5	11	-
Cycloheximide + α-bromonaphthalene[5] + DMSO[1]	3	28	-
Co419			
A) Isolated			
0.1% colchicine	4	11	-
0.1% colchicine	5	4	1
0.03% 8-hydroxyquinoline + DMSO[1]	3	4	-
1% α-bromonaphthalene	1	4	-
1% α-bromonaphthalene	2	6	-
B) Combined[2]			
Cycloheximide + colchicine[3] + α-bromonaphthalene[5*]	5	6	-
Colchicine[3] + 8-hydroxyquinoline	5	19	1
Cycloheximide + colchicine[3]	5	12	16
Cycloheximide + colchicine[3*]	5	9	-
Cycloheximide + 8-hydroxyquinoline	5	31	12
Cycloheximide + 8-hydroxyquinoline*	5	7	-
Cycloheximide + α-bromonaphthalene[5] + DMSO[1]	3	13	23
Cycloheximide + α-bromonaphthalene[5] + DMSO[1]	4	12	4

[1]2 drops DMSO per 10 ml of solution.
[2]Chemicals combined at the same concentrations used isolated.
[3]0.05% colchicine.
[4]0.009% cycloheximide + 0.025% 8-hydroxyquinoline.
[5]2% α-bromonaphthalene.
*Treatment at 15°C.

compared with number of metaphases selected, per treatment, for chromosome counting, according to the cell quality classification described below.

Root tips were fixed for 24 hours in 1:3 acetic-alcohol and kept in 70% alcohol at 10°C. Feulgen staining was used as follows: hydrolisis in 1N HCl at 60°C for 13 minutes, washing in distilled water, staining in Schiff's reagent for 45 minutes, and washing in tap water for 30 minutes. Then, the roots were macerated in a 2.5% pectinase plus 1% cellulase solution at 35°C for 5 minutes. Root tips were squashed in 1% acetocarmine. The cover-slips were removed in 45% acetic acid, and after air drying, the slides were mounted in Canada balsam. Prometaphase and metaphase cells were selected according to their quality and drawn with the aid of a camera lucida for chromosome counting. This procedure was different from that adopted by Tlaskal and Hutchinson (1974) who selected prophases with contracted chromosomes, as a guarantee to study intact cells. Cells clearly broken or with a high degree of clustered or overlapping chromosomes or with fuzzy chromosome morphology were discarded (Figure 1).

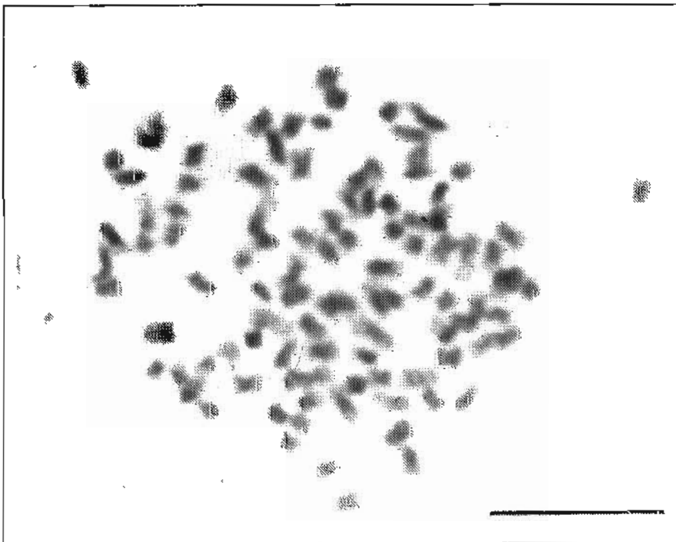


Figure 1 - Metaphase cell of variety NA56-79 (2n=114) pretreated with 0.007% cycloheximide plus 0.05% colchicine during three hours, showing fuzzy chromosome morphology, not used for chromosome counting. Bar equals 10 μm.

The accuracy of chromosome counts was evaluated through a cell quality classification procedure according to their visual appearance based on characteristics: 1) cell integrity; 2) degree of chromosome spreading; 3) details of chromosome morphology. Results of this classification were expressed in a numerical form, according to the scheme outlined in Table II, based on Tlaskal and Hutchinson (1974), with modifications. Cell integrity was checked in a phase contrast microscope. The classification of each cell was expressed as the sum of the values recorded for each characteristic. A metaphase graded seven corresponded to a cell clearly intact with a high degree of chromosome spreading and the morphology of most chromosomes clearly visible. The frequencies of each metaphase class were expressed in histograms showing the distribution of chromosome numbers found.

Table II - Classification of cell quality.

Rating	Cell integrity	Chromosome spreading	Detail of chromosome morphology
0	May not be entire		
1	Clearly entire	Considerable chromosome overlapping	Not visible on some chromosomes
2		Medium	Medium
3		Without chromosome overlapping	Clearly visible

RESULTS AND DISCUSSION

Combined treatments were more successful than the ones using isolated mitotic inhibitors (Table I). The treatment with the mixture of 0.025% 8-hydroxyquinoline plus 0.009% cycloheximide for five hours resulted in preparations with a higher number of metaphases suitable for chromosome counting in the variety NA56-79, as illustrated in Figure 2. The best metaphase preparations of the variety Co419 were obtained after a treatment with 0.007% cycloheximide plus 2% α-bromonaphthalene and DMSO for three hours (Table I, Figure 3). Some combinations of cycloheximide with either 8-hydroxyquinoline or colchicine also resulted in rather good preparations. Treatments with c-mitotic agents alone resulted in preparations with a higher frequency of cells with a high degree of chromosome overlapping or poor chromosome morphology (Figure 1).

Tlaskal (1980) studied the actions of cycloheximide and 8-hydroxyquinoline on dividing cells of root meristems of *Zea mays*. He observed differences in mitotic indices after treatment with each of these chemicals, due to the reduction of the frequency of all mitotic phases by 8-hydroxyquinoline, whereas cycloheximide reduced mitotic frequency, but produced only slight changes in the relative frequency of metaphases and prophases.

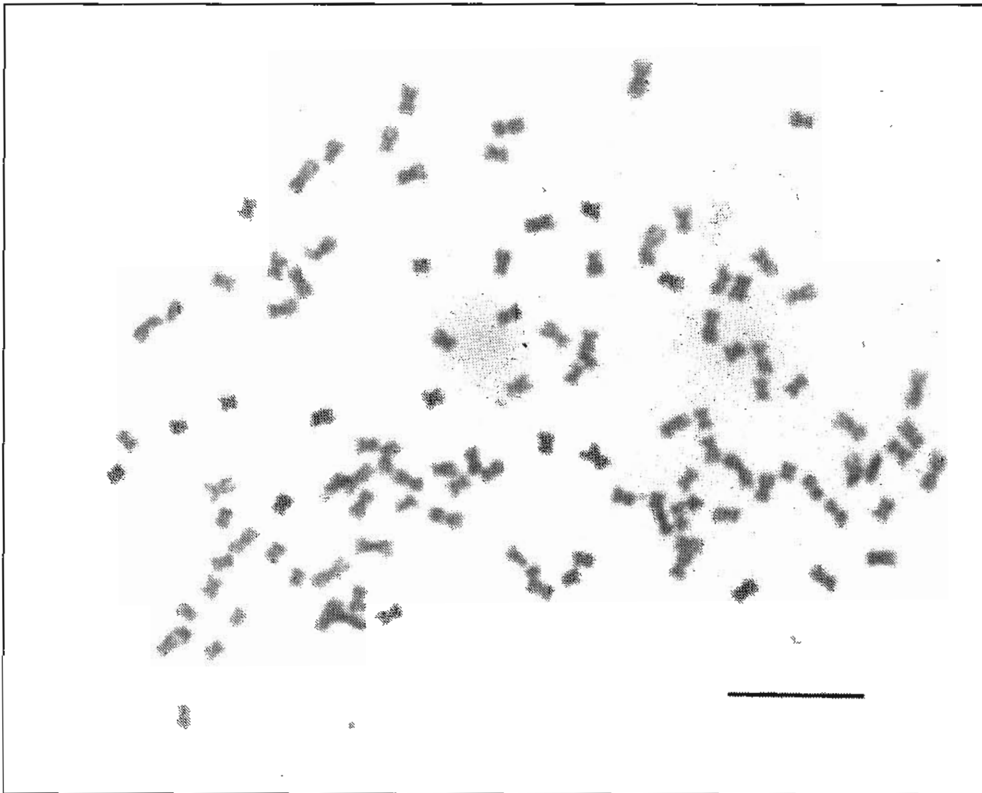


Figure 2 - Metaphase cell of variety NA56-74 (2n=114) pretreated with 0.009 cycloheximide plus 0.025% 8-hydroxyquinoline during five hours, with details of chromosome morphology clearly visible and no chromosome overlapping (cell classification = 6). Bar equals 10 μm.

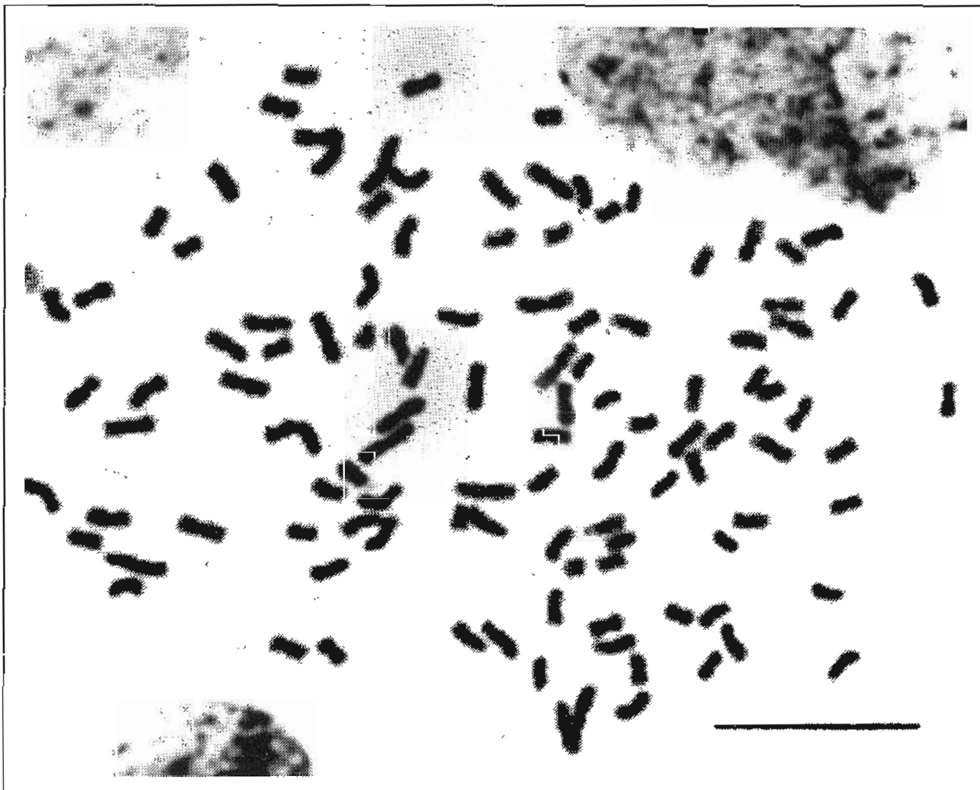
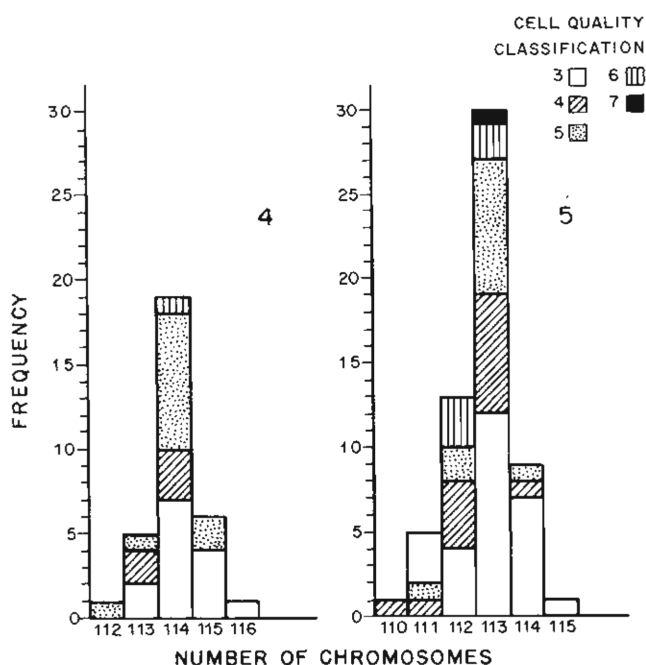


Figure 3 - Metaphase cell of variety Co419 (2n=113) pretreated with 0.007% cycloheximide plus 2% α-bromonaphthalene plus DMSO during three hours (cell classification = 7). Bar equals 10 μm.

In the frequency distribution of chromosome numbers counted, a modal number characteristic of each variety is quite clear (Figures 4 and 5). In variety NA56-79 the number was 2n-114, detected in 59.4% of the cells analysed, of which 47% had high quality (graded 5-6). In variety Co419, the modal number was 2n=113 (50,85% of the metaphases analysed) and 36.67% of these metaphases corresponded to the best cells observed (graded 5-7). In both varieties, the range in variability of chromosome numbers was low and could be considered as due to interpretation errors. Therefore, mosaicism does not occur in the materials studied. The results confirm a preliminary study of samples of NA56-79 pretreated with colchicine (Kunieda *et al.*, 1984). Frias *et al.* (1978) reported 2n=90-122 for NA56-79 and this variation seems to be due to problems of interpretation of the metaphases. The only report (Dutt and Rao, 1956) on the chromosome number of Co419 (2n=118) did not mention the methodology employed.



Figures 4 and 5 - Distribution of chromosome number counted. Figure 4, variety NA56-79. Figure 5, variety Co419. The frequencies of metaphases classes, graded from 3 to 7 according to their quality are expressed in the histograms.

In varieties in which chromosome mosaics clearly occur, the variability of chromosome counts is very high as in the case of the commercial sugarcane clone H50-7209 (2n=108-128) and derived somaclones (Heinz *et al.*, 1969). Tlaskal *et al.* (1970) and Tlaskal and Hutchinson (1973) also detected mosaics in some varieties and stated that the existence of major classes of chromosome numbers is evidence of the existence of subpopulations of cells ("stem lines"), each with a chracteristic chromosome number.

The varieties investigated here did not show somatic instability. The method that we described permitted the analysis of clearly intact metaphase cells, with the advantage that in this phase, the morphology of the chromosomes is quite clear.

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RESUMO

Durante uma investigação sobre a estabilidade do número de cromossomos de plantas regeneradas a partir de variedades de cana-de-açúcar, desenvolvemos uma metodologia para obtenção de preparações de metáfases mitóticas intactas, com aspectos da morfologia cromossômica bem nítidos e com baixa freqüência de cromossomos sobrepostos. Foram testados vários tipos de pré-tratamentos de pontas de raíz e as combinações de 8-hidroxiquinolina mais ciclohexímida e de α-bromonaftaleno com a mesma, produziram bons resultados, respectivamente, para as variedades NA56-79 (2n=114) e Co419 (2n=113). Foi estabelecido um critério para avaliação da precisão das contagens dos números de cromossomos e os resultados mostram que essas variedades apresentam estabilidade cromossômica.

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