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SHORT COMMUNICATION

Efficiency of Different Antimitotics in Cytological Preparations of Sugarcane

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Abstract Commercial sugarcane cultivars and wild species have a high ploidy level allied to a complex genome and usual chromosomal instability. There are few studies related to the standardization of cytogenetic techniques in sugarcane. This work tested the efficiency of three antimitotics (colchicine, 8-hydroxyquinoline, and trifuralin) at different concentrations on the quality of metaphases spreads from a commercial sugarcane cultivar (IAC911099-*Saccharum* spp.) and a wild sugarcane accession (Krakatau-*S. spontaneum*). Although all three antimitotics were efficient for obtaining metaphases, 8-hydroxyquinoline was the most efficient for both IAC911099 and Krakatau. The chromosome number of these two genotypes was inferred. The variation in chromosome number was 2n = 90–112 for IAC911099, with a statistical modal of 2n = 112 chromosomes while the variation in chromosome number was 2n = 90–129 chromosome with a statistical modal of 2n = 128 chromosomes for Krakatau (*S. spontaneum*).

Keywords Sugarcane · Colchicine · 8-Hydroxyquinoline · Trifuralin · Cytogenetics

Sugarcane is an important crop worldwide due to sugar, ethanol, and more recently, biomass production. Native to Southeast Asia, sugarcane belongs to the Poaceae family and *Saccharum* genus, which includes six main species: *S. officinarum, S. spontaneum, S. robustum, S. sinense, S. barberi* and *S. edule.*

Modern sugarcane cultivars, *Saccharum* spp., are hybrids from crosses between *S. officinarum,* the high sugar content species, and *S. spontaneum* (D'Hont et al. 1996), followed by several backcrosses to *S. officinarum* (the noble parent) to recover sucrose content. This nobilization process has contributed to the high number of chromosomes found in todays cultivars (Cuadrado et al. 2004).

Studies related to the standardization of chromosome preparations and chromosome numbers in sugarcane are relatively few. The main difficulties in sugarcane cytogenetic studies are the high ploidy level added to the complex genome, aneuploidy, chromosomal instability (Grivet and Arruda 2002), and the high number and small size of the chromosomes (D'Hont et al. 1998). In addition, there is a lack of one concise technique to count of the species' true chromosome numbers (Silvarolla and Aguiar-Perecin 1994; Cuadrado et al. 2004). However, several methods to obtain good metaphases have been well described for sugarcane (Silvarolla and Aguiar-Perecin 1994; D'Hont et al. 1996).

Today, many laboratories have established their own methodology for counting sugarcane chromosomes, but most of them are not published. Hence information on new pretreatments and techniques to obtain good metaphases is not easily available.

Using different antimitotics in cytogenetic preparations is an approach to optimize and establish new cytogenetic protocols. According to Guerra and Souza (2002), the ideal antimitotic has blocker capacity in the metaphase mitotic cycle, which leads to a high contraction and good spreading in the chromosome preparation due to the inhibition or

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destruction of the mitotic spindle. Colchicine, 8-hydroxyquinoline, and trifuralin are among the most commonly used antimitotics.

In the past, colchicine was extracted from the *Colchicum sativum* plant, but today it is manufactured synthetically (Mondin and Neto 2006). This antimitotic blocks the spindle formation in the prophase as a consequence of protein polymerization impediment and delayed the separation of chromosomes, making the metaphase chromosome visualization easier (Elgsti and Dustin 1955). It can also be used for ploidy induction (at high concentrations) or as a pharmaceutical at low concentration.

Trifuralin is an herbicide from the dinitroaniline family and is used in crops such as soybeans, citrus, and cassava. It acts mainly in the tubulin protein by depolymerizing the microtubule and stopping cell division (Morejohn and Fosket 1991).

The final antimitotic is 8-hydroxyquinoline, a drug from the quinolone family. It promotes significant spindle inactivation followed by chromosome concentration in the cell equatorial plan.

In this work, we studied the efficiency of these three antimitotics at different concentrations for the number of pro-metaphase and metaphase cells as well as of the visual quality of the cell (without scoring), using a commercial sugarcane cultivar (IAC911099-*Saccharum* spp.) and a wild sugarcane accession (Krakatau-*S. spontaneum*). After choosing the best antimitotic pre-treatment, the chromosome number of these two genotypes was inferred.

Stalks from IAC911099 and Krakatau (*S. spontaneum*) were collected from the Experimental Station of the Instituto Agronômico (IAC–Sugarcane Center) located at Ribeirão Preto, São Paulo, Brazil. Buds were cut from the stalks and placed in a plastic tray filled with *Sphagnum.* They were watered and placed in a germination chamber (B.O.D.) at 35 °C until roots were formed (1.5 cm long). Roots of similar sizes were collected and immediately immersed in each of the different antimitotics (colchicine, 8-hydroxyquinoline and trifuralin) for 4 h at room temperature. Each antimitotic was evaluated at two concentrations: 0.1 and 0.05 % (colchicine), 0.04 and 0.02 % (8-hydroxyquinoline), and 3 and 9 μM (trifuralin).

After the pre-treatment the roots were washed and fixed in Farmer's solution (alcohol:acetic acid fixative, at a ratio of 3 ethanol:1 acetic acid) and stored at 4 °C. The roots were then washed again with distilled water and hydrolyzed in 1 N HCl at 60 °C for 12 min. Ten slides were observed for each antimitotic at each concentration. The number of cells in pro-metaphase and metaphase was recorded, and the visual quality (without scoring) was assessed. The data were transformed using the Neperian logarithm, and the analysis of variance and mean comparison (LSD-Least Significant Difference) was performed on SAS statistic software (SAS Institute 2008).

To estimate the chromosome numbers of IAC911099 and Krakatau, the chromosome count was done after the selection of the best antimitotic and respective concentration. In this case, each pro-metaphase and metaphase received a score according to visual observation. The pro-metaphases and metaphases were classified as good (high degree of chromosomic spreading and condensation); medium (good condensation with chromosomes overlapping); and bad (low degree of spreading, chromosome overlapping, and low chromosome condensation), receiving scores 3, 2, and 1, respectively. This was done because a metaphase with a high score certainly will provide a more reliable chromosome number.

Thirty-six metaphases and pro-metaphases were selected for each genotype. The metaphase quality scores weighted the number of chromosomes [(number of chromosome counted in the respective metaphase) x (score)]. The metaphases were observed in an optical microscope (1000x) and the chromosome count was performed on IKAROS by Metasystems. The metaphase images were captured on an Axiocam 5 s using the Axiovision 4.8 program from Carl Zeiss Vision. Statistical data such as mode and weight average were also estimated.

All antimitotics were efficient for both genotypes (IAC911099 and Krakatau), since no mitotic phases after metaphase were observed.

When the averages for both concentrations of the individual antimitotics were compared, 8-hydroxyquinoline had the best results ($P < 0.05$) for both genotypes (Table 1). In addition, 8-hydroxyquinoline also produced metaphases of high superior quality (Figs. 1, 2, 3, 4). According to the literature (Silvarolla and Aguiar-Perecin 1994; Cuco et al. 2003), the same antimitotic can produce different results in different species and cultivars. In our work, 8-hydroxyquinoline was effective for both the genotypes evaluated.

When the analysis of variance was performed, no significant difference ($P < 0.001$) was observed between the antimitotic concentrations for either trifluralin or colchicine in cultivar IAC911099 (Table 2). On the other hand, for this same cultivar, the two 8-hydroxyquinoline concentrations were significantly different ($P < 0.001$) (Table 2), with the 0.04 % concentration more effective than the 0.02 % (Table 3). Although the antimitotic concentrations did not differ significantly for the number of pro-metaphases and metaphases for Krakatau (Table 2), the 8-hydroxyquinoline at 0.04 % had the highest mean (Table 3).

Metaphases of both genotypes obtained by 8-hydroxyquinoline exhibited good chromosome spread and better visual quality (Figs. 1, 2, 3, 4). This pre-treatment also

Table 1 *T*-test (LSD) showing the statistical difference between the means of the number of pro-metaphases and metaphases obtained at both concentrations of different antimitotics in *Saccharum* spp. (IAC911099) and *S. spontaneum* (Krakatau)

Methods	8-hydroxyquinoline		Trifuralin		Colchicine	
Cultivars	IAC911099	Krakatau	IAC911099	Krakatau	IAC911099	Krakatau
Mean	2.312[a]	2.075[a]	1.813[b]	1.795[b]	1.683[b]	1.809[b]
N	20	20	20	20	20	20

Means with the same letter are not significantly different ($P > 0.05$); *N* total number of slides considering both concentrations (10 slides for each antimitotic concentration)

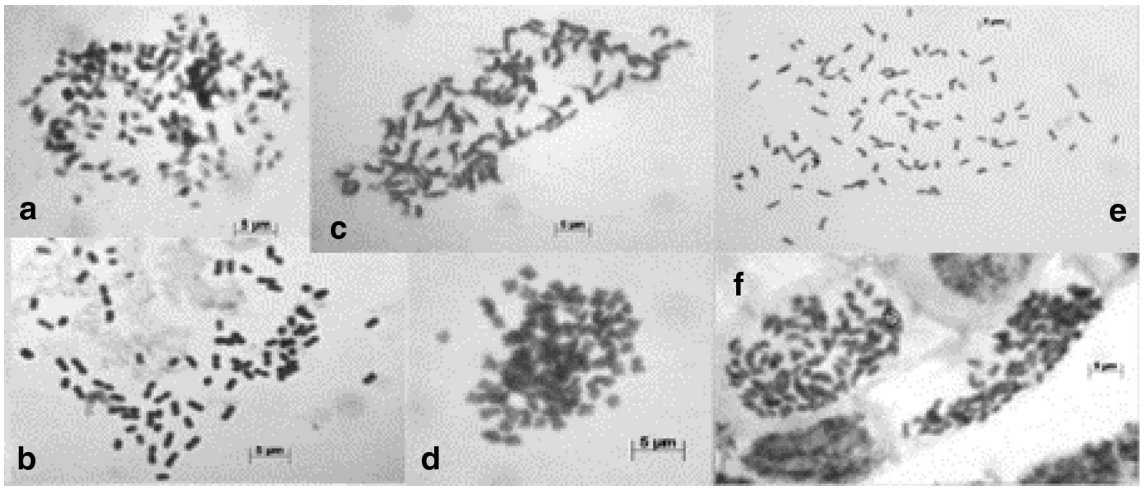

Fig. 1 Metaphase or pro-metaphase of IAC911099 obtained in **a** colchicine 0.1 %; **b** colchicine 0.05 %; **c** trifuralin 9 μM; **d** trifuralin 3 μM; **e** 8-hydroxyquinoline 0.04 %; **f** 8-hydroxyquinoline 0.02 %

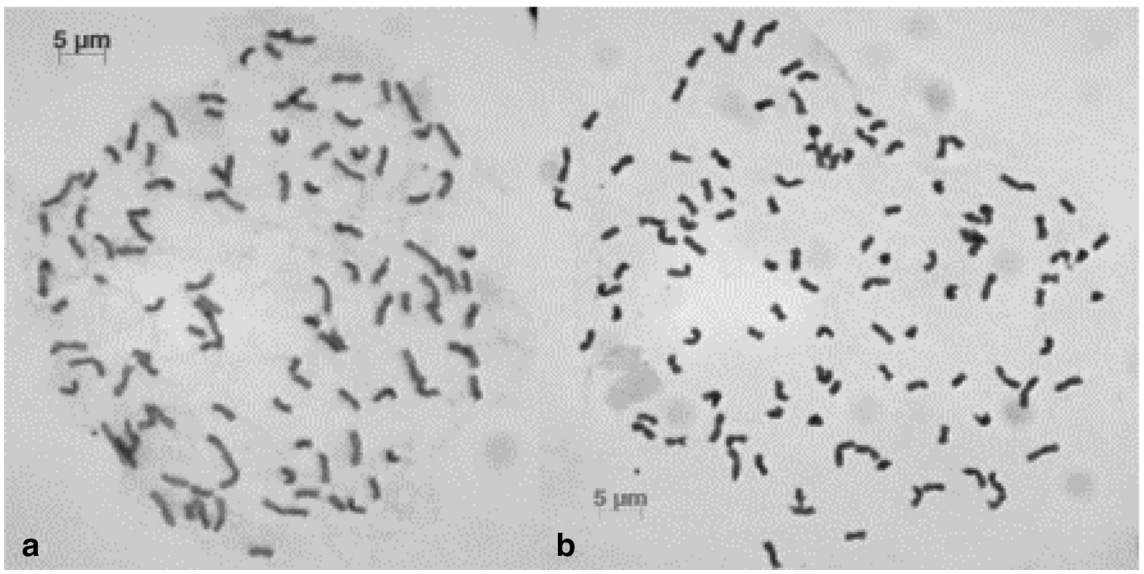

Fig. 2 Metaphase of cultivar IAC911099 in 8-hydroxyquinoline 0.04 % **a** 2n = 112 chromosomes; **b** 2n = 112 chromosomes

produced similar chromosome condensations; reflecting a better stability. The 0.04 % concentration produced chromosomes in both genotypes that were more defined than the 0.02 % concentration (Figs. 1, 3). The low 8-hydroxyquinoline concentration (0.02 %) led to a smaller number of metaphases and a high number of pro-metaphases and final prophases when compared to the higher concentration.

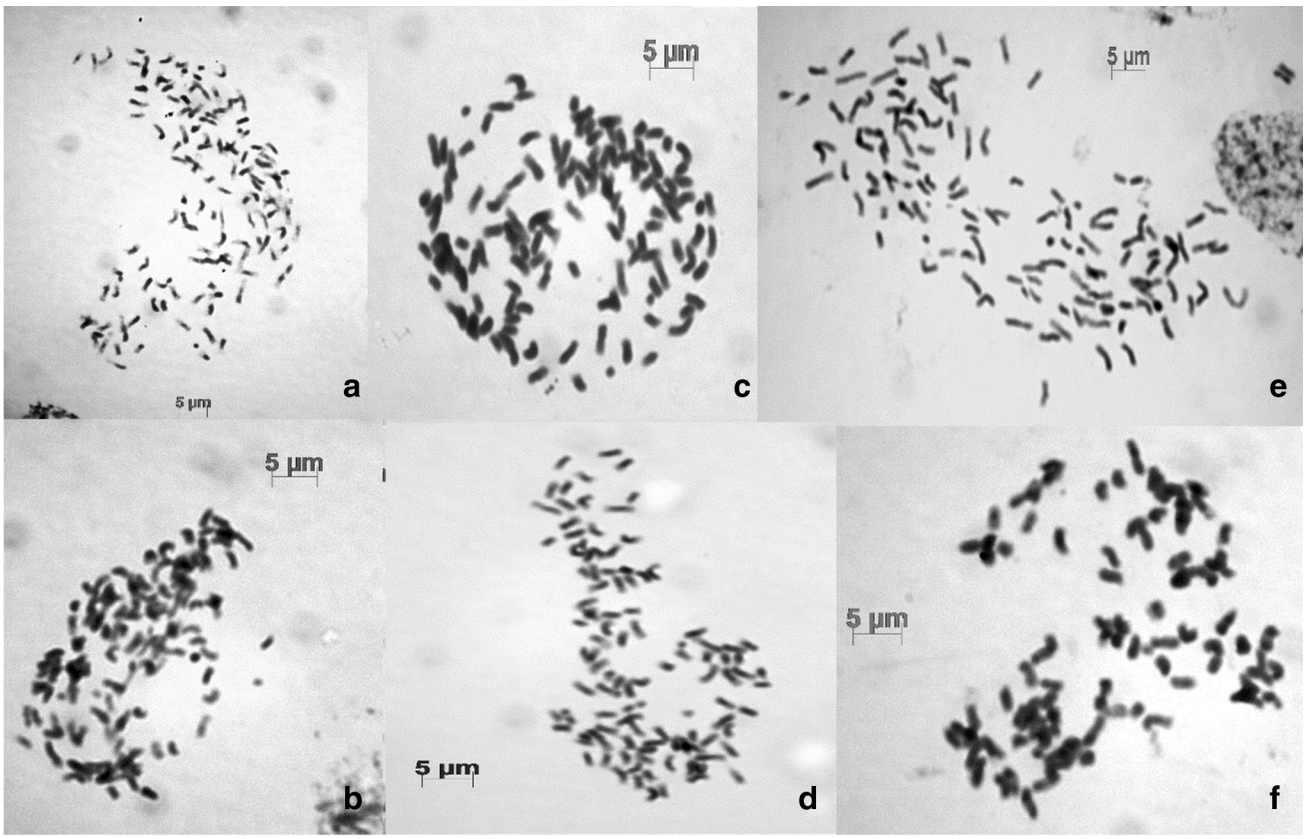

Fig. 3 Metaphase or pro-metaphase of Krakatau obtained in **a** colchicine 0.1 %; **b** colchicine 0.05 %; **c** trifuralin 9 µM; **d** trifuralin 3 µM; **e** 8-hydroxyquinoline 0.04 %; **f** 8-hydroxyquinoline 0.02 %

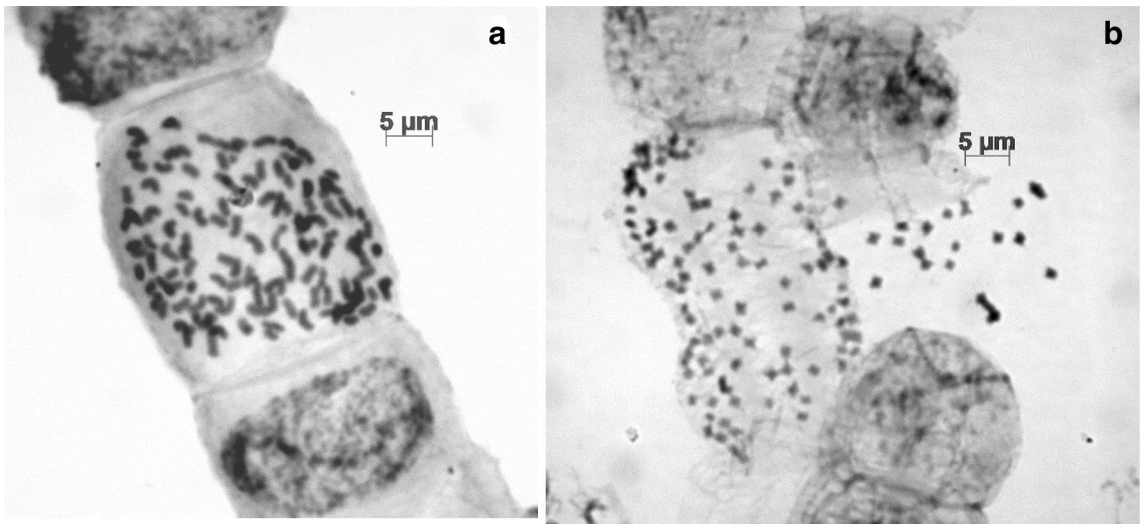

Fig. 4 Mitotic metaphase of Krakatau (*S. spontaneum)* in 8-hydroxyquinoline 0.04 % **a** 2n = 128 chromosomes; **b** 2n = 128 chromosomes

Although the use of colchicine as an antimitotic was once common among both plant and animal cytogenetic studies (Cardoso et al. 2012; Gupta et al. 2013; Bonasora et al. 2013), in plant research today, it is being replaced by other antimitotics. Trifluralin may be an alternative to colchicine; however, its use as an antimitotic in sugarcane cytological preparations of mitotic metaphases has not yet been reported in the literature. Due to its lower cost and

Table 2 Analysis of variance for different antimitotics and the number of pro-metaphases and metaphases of *Saccharum* spp. (IAC911099) and *S. spontaneum* (Krakatau)

Method	DF	IAC911099			Krakatau		
		Mean squares	F value	Pr > F	Mean squares	F value	Pr > F
8-Hydroxyquinoline	1	2.172	33.24	< 0.001	0.071	0.52	0.472
Trifuralin	1	0.031	0.48	0.491	0.219	1.6	0.211
Colchicine	1	0.001	0.03	0.873	0.060	0.44	0.508

DF degree of freedom

Table 3 Mean and adjusted standard deviation of pro-metaphases and metaphases of *Saccharum* spp. (IAC911099) and *S. spontaneum* (Krakatau) with different antimitotics and their respective concentration

Concentration	Method	N	IAC911099		Krakatau	
			Mean	Standard deviation	Mean	Standard deviation
0.02 %	8-hydroxyquinoline	10	1.983	0.308	2.011	0.475
0.04 %	8-hydroxyquinoline	10	2.642	0.328	2.131	0.524
0.1 %	Colchicine	10	1.674	0.153	1.864	0.325
0.05 %	Colchicine	10	1.692	0.155	1.753	0.210
3 μM	Trifuralin	10	1.852	0.320	1.690	0.172
9 μM	Trifuralin	10	1.773	0.197	1.899	0.380

N number of slides

toxicity, trifluralin should be investigated to improve its use as an alternative pre-treatment (Mondin and Neto 2006).

Often, for cytogenetic studies, a combination of different antimitotics is used. This would increase the cost of the study, as some drugs are not marketed in all countries because of high commercial price and toxicity. The use of 8-hydroxyquinoline has been increasingly used in plant species and may be a possible replacement for colchicine (Mondin and Neto 2006). Today, 8-hydroxyquinoline at 0.04 % is widely used in sugarcane (D'Hont et al. 1996, 1998, 2002; Cuadrado et al. 2004).

Silvarolla and Aguiar-Perecin (1994), working with the sugarcane cultivar NA56-79, reported good quality metaphases when use colchicine with cycloheximamide. The combination of 8-hydroxyquinoline and cyclohexamide allows metaphase accumulation and a high number of final prophases, thereby facilitating karyotyping (Cuco et al. 2003). In this work, the use of 8-hydroxyquinoline alone produced this same effect.

The exposure of the roots to the antimitotic for 4 h showed good results and favored an increase of chromosome preparations. The number of pro-metaphases and metaphases for IAC911099 at the 0.04 % 8-hydroxyquinoline concentration ranged from 5 to 21 per slide, while the 0.02 % ranged from 0 to 8 per slide. The number of pro-metaphases and metaphases for this same cultivar with trifuralin ranged from 0 to 3 and from 0 to 6 per slide for the 9 and 3 μM concentrations, respectively. For colchicine, the number of pro-metaphases and metaphases ranged from 0 to 3 per slide for both the 0.1 and 0.05 % solution.

The number of pro-metaphases and metaphases observed for Krakatau at 0.04 % of 8-hydroxyquinoline ranged from 0 to 20, while 0.02 % was from 0 to 15. For trifuralin, the range was 0–10 for the 9 μM concentration and 0–3 for the 3 μM concentration. For colchicine, the range was 0–7 for the 0.1 % concentration and 0–2 for the 0.05 % concentration.

The coefficient of variation (CV) was 13.2 % for IAC911099 and 19.6 % for Krakatau. This large difference may be due to the widely varying ranges for the number of metaphases per slide, possibly caused by the cultivars' inherently different physiologic conditions of root growth. Moreover, cell counting by slide produces values having a Poisson distribution, in which the expected mean and variance are equal (have the same values), thereby generating a high variation coefficient (Perecin and Barbosa 1994).

The variation in the number of chromosomes for IAC911099 was 2n = 90–112 and the statistical mode was 2n = 112 chromosomes (36 % of metaphases) (Figs. 2, 5). Approximately half of the observed metaphases showed 2n = 110–112 chromosomes. For Krakatau (*S. spontaneum*), the range of counted chromosomes was

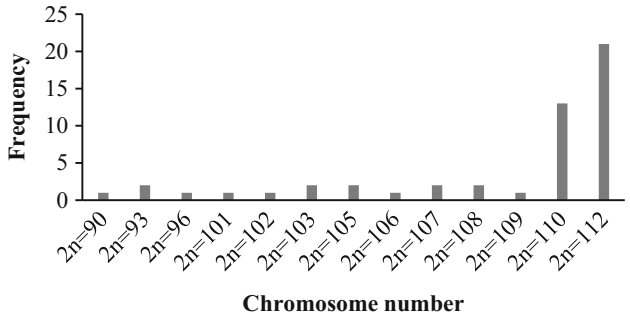

Fig. 5 Chromosome number variation in sugarcane cultivar IAC911099

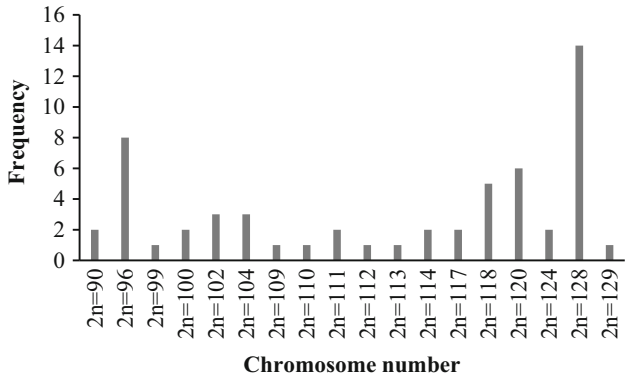

Fig. 6 Chromosome number variation in Krakatau (S. *spontaneum*)

2n = 90–129 and the statistical mode was 2n = 128 (38 % of metaphases) (Figs. 4, 6). Furthermore, in this genotype 2n = 96 chromosomes (15 % of metaphases) was also found.

The high number of chromosomes, the small size, and the similarity between the chromosomes are all factors that influence chromosomal analysis of the *Saccharum* genus (D'Hont 2005). However, variation in sugarcane chromosome numbers is usually caused by either the difficulty in obtaining intact cells—without chromosomes lost, or the wrong interpretation of overlapping chromosome counts (Tlaskal and Hutchinson 1973; Silvarolla and Aguiar-Perecin 1994), although the existence of aneuploidism cannot be ruled out (D'Hont et al. 2002).

Despite this, the variation in the number of chromosome observed for IAC911099 in our study is within the expected range (2n = 100–130) of modern sugarcane cultivars (D'Hont et al. 1996). Silvarolla and Aguiar-Perecin (1994) reported 2n = 114 chromosome for NA56-79 and 2n = 113 chromosome for Co419, two important sugarcane cultivars wildly planted in Brazil in the past. A variation of chromosomes of 2n = 107–115 was reported for the R570 cultivar (D'Hont et al. 1996), and 2n = 112 chromosomes for NCo376 (D'Hont 2005). Other authors have found similar variation for sugarcane cultivars from different sugarcane breeding programs (Barreto and Simon 1982; Jenkin et al. 1995; Cuadrado et al. 2004).

In relation to *S. spontaneum*, a wide variation in chromosome numbers (2n = 40–2n = 128), including a high number of cytotypes (2n = 64, 2n = 80, 2n = 96, 2n = 112, and 2n = 128) has also been reported (Panje and Babu 1960). These numbers are in agreement with D'Hont et al. (1998), who confirmed the basic chromosome number of x = 8 chromosome for this specie. This same author observed low chromosome numbers of 2n = 64 for the *S. spontaneum* acessions SES 14 and SES 106B, as well as 2n = 80 for Mol 5801 and NG 51-2, but a high number of chromosomes for Mandalay (2n = 96) and Glagah 1286 (2n = 112). These later findings are in agreement with the variation in the chromosome numbers found in our study for Krakatau.

In our work, although all three antimitotics were efficient for obtaining metaphases, the 8-hydroxyquinoline was the most efficient for producing good results for both IAC911099 and Krakatau.

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