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In vivo HAPLOID INDUCTION AND EFFICIENCY OF TWO CHROMOSOME DUPLICATION PROTOCOLS IN TROPICAL MAIZE

Indução *in vivo* de haploides e eficiência de dois protocolos de duplicação cromossômica em milho tropical

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ABSTRACT

Artificial chromosome duplication is one of the most important process in the attainment of doubled haploids in maize. This study aimed to evaluate the induction ability of the inducer line KEMS in a tropical climate and test the efficiency of the R1-Navajo marker by flow cytometry to evaluate two chromosome duplication protocols and analyze the development of the doubled haploids in the field. To accomplish this goal, four genotypes (F1 and F2 generations) were crossed with the haploid inducer line KEMS. The seeds obtained were selected using the R1-Navajo marker and subject to two chromosome duplication protocols. Duplication was confirmed using flow cytometry. The percentages of self-fertilized plants after duplication as well as the quantities of doubled haploid seeds obtained after the self-fertilization processes were analyzed. It was observed that the germplasm influences haploid induction but not the duplication rates of the tested protocols. Protocol 2 was more efficient for the duplication of haploids, in the percentage of self-fertilized plants after duplication, and in the attainment of doubled haploid lines. Moreover, the haploid inducer line KEMS can produce haploids in a tropical climate. Other markers, in addition to the R1-Navajo system, should be used in the selection of haploid seeds.

Index terms: R-Navajo; colchicine; induction of maternal haploids; *Zea mays.*

RESUMO

A duplicação cromossômica artificial está dentre as etapas mais importantes na obtenção de duplo-haploides em milho. Este estudo objetivou avaliar a capacidade de indução da linhagem indutora KEMS em clima tropical e testar a eficiência do marcador R1-navajo por meio de citometria de fluxo; avaliar dois protocolos de duplicação cromossômica e, analisar o desenvolvimento dos haploides duplicados no campo. Para isso, quatro genótipos (gerações F1 e F2) foram cruzados com a linhagem KEMS. As sementes obtidas foram selecionadas pelo marcador R1-navajo e submetidas a dois protocolos de duplicação cromossômica. A duplicação foi confirmada por meio de citometria de fluxo. As porcentagens de plantas autofecundadas após duplicação foram analisadas, bem como as quantidades de sementes duplo-haploides obtidas após as autofecundações. Foi observado que o germoplasma influencia a indução de haploides, mas não na taxa de duplicação dos protocolos testados. O protocolo 2 foi mais eficiente na duplicação de haploides, na porcentagem de plantas autofecundadas após duplicação, e na obtenção de linhagens duplo-haploides. Além disso, a linhagem indutora KEMS pode induzir haploides em clima tropical. Outros marcadores além do sistema do R1-navajo devem ser utilizados na seleção de sementes haploides.

Termos para indexação: R-navajo; colchicina; indução de haploides maternos; *Zea mays*.

INTRODUCTION

Doubled haploid technology has been used in breeding programs for several decades in various species (Li et al., 2013). Various private companies produce doubled haploid lines for the attainment of hybrid maize. However, information concerning the efficiency of the technique in public institutions is still scarce.

The implementation of the doubled haploid method in maize requires the use of haploid inducer lines. Generally, these haploid inducer lines have a temperate origin, which hampers the handling and development of these lines in tropical conditions. According to Rotarenco et al. (2010) and Dang et al. (2012), some haploid inducer lines have been developed and have relatively high haploid induction rates, such as: MHI with 7.2% induction, the modern European line RWS with 8% induction, and the

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PHI line with 12.8% induction. However, some limiting factors exist in the haploid induction technique such as the haploid frequency may be influenced by the hybrid used as the donor (Kebede et al., 2011; Prigge et al., 2011).

Haploids obtained by inducer lines are selected using a morphologic marker based on the anthocyanin pigmentation in the seed endosperm. This pigmentation is controlled by the R1-Navajo gene (Chase; Nanda, 1965). This gene shows dominant allelic interactions. In addition, R1-Navajo expression can be reduced due to the female parent because it can carry known inhibitor genes (Melchinger et al. 2014). Other morphological markers have been used to select haploids such as plant height, plant vigor, and the absence of ligule (Prigge et al., 2012). Moreover, other techniques such as flow cytometry and molecular markers have also been used (Wu et al., 2014; Couto et al., 2015).

As one of the most important stages in the attainment of the doubled haploids, artificial chromosome duplication is noteworthy. Few methods of chromosome duplication have been tested and published (Eder; Chalik, 2002; Castillo et al., 2009; Häntzschel; Weber, 2010). Duplication protocols using antimitotics have been used increasingly, because they promote the chromosomic duplication/doubling and the attainment of double-haploid strains that are completely homozygous. Castillo et al. (2009) suggested that time, genotype, and concentration factors influence chromosome duplication when colchicine is used. However, information concerning the behavior and efficiency of chromosome duplication requires further research.

In that context, the objectives of this study were a) to evaluate the induction capacity of the haploid inducer line KEMS in a tropical climate and test the efficiency of the R1-Navajo morphological marker using flow cytometry and b) to evaluate two protocols of chromosome duplication and analyze the development of the duplicated haploids in the field.

MATERIAL AND METHODS

Evaluated hybrids

The haploid inducer line KEMS (Shatskaya et al., 1994) was used as a pollen donor and crossed with four simple hybrids (DKB393, GNS 3225, GNS 364, and GNS 3032) and their F2 generations. The cross field test occurred in Cravinhos, in the State of São Paulo (650 m altitude and 21°20'25'S, 47°43'46'W), in the second season of 2011. The culture treatments were conducted according to recommendations for maize culture.

Identification of haploids using the R-Navajo marker

To obtain probable haploids, the seeds obtained with the previously cited crosses were visually separated according to the purple color of the endosperm and the white color of the embryo due to the R1-Navajo marker (Chase; Nanda, 1965). The seeds of the probable haploids were further divided and subjected to two different chromosome duplication protocols. The total quantity of the seeds selected as haploids according to the R1-Navajo marker was used in the statistical analyses for the calculation of the haploid induction rate (HIR) of the KEMS line.

Artificial chromosome duplication

Two chromosome duplication protocols were used. For Protocol 1, maize seedlings (five days after germination) were subjected to treatment with a 0.04% colchicine solution and 0.5% dimethyl sulfoxide (DMSO) for 12 hours and kept in the dark at 20 ºC (Prasanna et al., 2012). For Protocol 2, the selected seeds were sown in trays containing sand and vermiculite (ratio 1:1). After 10 days, the roots of the plants were washed in water, immersed in 0.1% colchicine solution, 0.1% DMSO, and 0.1% Tween 20 for six hours in the presence of light at ambient temperature (approximately 22 ºC) according to the method of Paul Newell (non-published data).

After the duplication process, the seedlings and roots subjected to the colchicine treatment, in the two protocols, were washed for 40 minutes with running water and transferred to a greenhouse where they were maintained for 20 days.

Flow cytometry

At fourteen days after chromosome duplication, leaf samples were collected and analyzed with a flow cytometer to identify duplicated plants and evaluate of the efficiency of the duplication protocols.

The estimate of the quantity of DNA was obtained from the leaf tissue of the parents and the descendants originating from that cross that survived chromosome duplication. For each sample, approximately 20-30 mg of young leaves of the evaluated individual were used. The *Vicia faba* species (DNA quantity 26.9 pg/2C) was used as an external reference standard. The samples were ground in a Petri dish containing 1 mL of cool LB01 buffer, according to the method of Dolezel (1997), for the attainment of the nuclear suspension, to which 2.5 µL of RNase was added, and the samples were stained with 25 µL of propidium iodide (1 mg mL^{-1}). For each sample,

a minimum of 10,000 nuclei were analyzed. Histograms were obtained using a *FacsCalibur* (*Becton Dickinson*) cytometer with the *Cell Quest* (Becton, Dickinson and Company, San Jose, CA, USA) program and analyzed using the WinMDI 2.8 software (2009).

Evaluations of self-fertilization and quantities of seeds obtained from duplicated plants

After 20 days of post-chromosome-duplication acclimatization and after flow cytometry analysis, plants that underwent both protocols were transplanted to a protected environment at the HortiAgro company, located in the municipality of Ijaci, MG (830 m altitude and 21°9'24'S, 44°55'34'W). All plants that produced pollen and that had a synchronized style and stigma were self-fertilized for the maintenance and multiplication of seeds.

Information about the self-fertilized plants was collected, and tracking of the ploidies was conducted via flow cytometry. Moreover, the quantity of seeds obtained after the self-fertilization processes and the quantity of the seeds of the doubled haploids were analyzed.

Statistical analysis

Statistical analyses were performed to evaluate the induction capacity of the haploid by R-Navajo and by flow cytometry, artificial chromosome duplication, and quantity of seeds obtained from duplicated plants, according to the method described by Basttistelli et al. (2013). All experiments were performed without replicates. The generation was used as the replication, and the ratios observed were evaluated using the generalized linear mixed model approach (GLMM) because overdispersion was detected (Nunes; Morais; Bueno Filho, 2004). In this case, the binomial GLMM was employed as a *logit* link according to the following description in which the protocol analysis is used as an example:

$$\frac{R_{ijk}}{p_{ijk}} \sim \frac{Binomial\left(m_{ijk}, \pi_{ijk}\right)}{m_i}$$

$$\log\left(\frac{\pi_{ijk}}{1-\pi_{ijk}}\right) = \mu + p_i + h_j + g_k + ph_{ij} + p_{ijk}$$

where R_{ijk}/p_{ijk} is the ratio observed in the portion of plants that underwent protocol i for hybrid j in generation k admitted conditionally, independent of the random effect of the portion; μ is the intercept; p_i is the fixed effect of protocol i; h_j is the fixed effect of hybrid j; g_k is the fixed effect of generation k; ph_{ij} is the fixed effect of the interaction of hybrid j with protocol i; and p_{ijk} is the random effect of portion ijk, where pijk ~ N (0,).

The GLMM was adjusted with estimation of the fixed and random effects, as well as the variance components, via the restricted maximum likelihood (REML, Patterson and Thompson, 1971) using the lme4 pack of the R program (Bates; Maechler; Bolker, 2014).

The significance of the effects of the model was verified via *deviance* analysis with the application of the χ^2 (cui-square) statistical test with a 5% probability. For the effects where significant differences were evident, a grouping was performed based on the *Mahalanobis* distance using the nearest neighbor method, and the cut-off point was established using the *bootstrap* resampling method.

RESULTS AND DISCUSSION

Identification of haploids using the R1-Navajo marker

The quantities of seeds obtained from the crosses among the inducer line KEMS and the simple hybrids GNS 3032, GNS 3225, GNS 364, and DKB 393 and their F2 generations are shown in Table 1. The probable haploids were selected from these seeds according to the R1-Navajo system marker (Chase; Nanda, 1965).

Using the *deviance* analysis, it can be observed that the different hybrids influence the HIR when using the R1-navajo morphologic marker (Figure 1A). No significant differences were found in the HIR between the generations of hybrids used. These results

Table 1: Total of seeds obtained (T) and quantities of seeds classified as haploids selected by the R1-Navajo morphologic marker (Q) from different hybrids and generations of maize.

Hybrids	Generations	T	Q
GNS3225	F1	2236	126
	F2	2284	145
GNS3264	F1	2523	241
	F2	1450	135
GNS3032	F1	1193	177
	F2	238	47
DKB393	F1	3486	130
	F2	1904	85

corroborate those observed by Battistelli et al. (2013), who demonstrated that a generation of endogamy does not interfere with the HIR.

Differences between genotypes and the HIR in maize have been reported by several authors (Battistelli et al., 2013; Eder; Chalyk, 2002; Rober; Gordillo; Geiger, 2005). The average HIR of the selected haploids using the R1-Navajo marker was 8.5%. Battistelli et al. (2013), using the same haploid inducer line, obtained an average induction rate of 7.1%. Shatskaya et al. (1994) obtained values varying from 6.3% to 8%. Differences between the hybrids for the HIR of maize haploids have previously been reported by other authors (Kebede et al., 2011; Wu et al., 2014). According to Belicuas et al. (2007), these rate differences occur due to the variable expressivity of the R1-Navajo gene, which does not produce precise indication of the seeds.

According to Rotarenco, Dicu and Sarmaniuc (2009), induction rates may depend on the method used in the crosses and the pollination period. Better results were obtained when the authors used manual pollination than when open pollination was used. However, Geiger and Gordillo (2009) cite that experienced breeders can obtain a higher HIR using non-controlled pollination processes. Moreover, according to Kebede et al. (2011), the environmental conditions interfere with the HIR.

Haploid identification by flow cytometry

Haploids were classified as those individuals who present a characteristic peak of ploidy of x in the histograms, according to Battistelli et al. (2013), independent of whether the samples have undergone duplication. Using this analysis, it was possible to verify the efficiency of the R1-Navajo marker.

In the *deviance* analysis, a significant difference was found between the hybrids. In the grouping based on the *Mahalanobis* distance, however, the averages were the same, that is, at a probability of 5%, the hybrids did not differ in the real haploid rate (Figure 1B).

Considering the HIR observed in this study, it is perceived that the actual percentage of haploid induction was lower than that obtained using the R1-Navajo marker. Thus, it is possible to verify that the R1-Navajo marker is inefficient and that other markers need to be used. Regarding the averages, an error rate of 33.5% was observed because the average of the haploids identified by the R1-Navajo marker was 8.50%, whereas the average of the haploids identified by flow cytometry was 2.85% (Figures 1A and 1B).

As described by Belicuas et al. (2007), the R1-navajo gene presents variable expressivity, and thus, the marker does not provide a precise indication of the seeds, which allows false haploids to be selected. To avoid this error, Prigge et al. (2011) recommended analyzing and separating the haploid seeds after the harvest because the drying stage generates a darker color of the embryo, contributing to erroneous selection. Moreover, other morphologic markers may be used in the detection of the false haploids, such as the color of the stem, ligule, and vertical leaves involving the stem (Prigge et al., 2012).

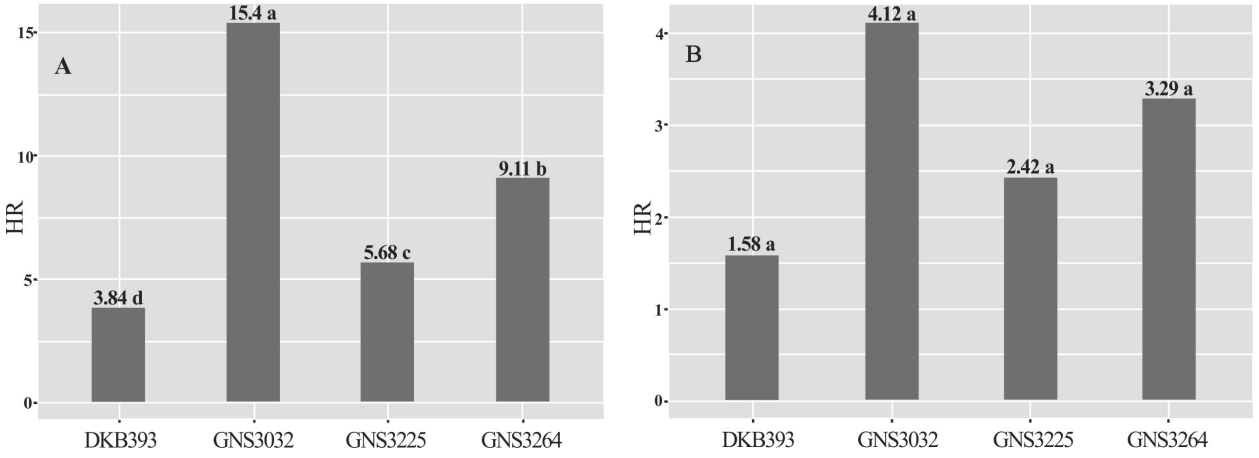

Figure 1: Mean percentages of haploid induction using the R1-Navajo marker (A) and haploid identification by flow cytometry (B). Mean percentages followed by the same letter belong to the same cluster. The values are based on the Mahalanobis distance with a cutoff of 5% probability.

Chromosome duplication of haploid lines

First, it is necessary to emphasize that these analyses were conducted considering the number total of plants that survived the duplication stage and were analyzed by flow cytometry. The total number of plants that were subjected to the duplication protocols was not considered. In other words, the duplication analyses were conducted on a total number of seeds that was smaller than the total number of plants that were initially included in the study because various seedlings did not survive the colchicine treatment.

Thus, in the *deviance* analysis of the total number of duplicated plants, the protocols differed significantly between each other, as well as between the generations. The adjusted averages obtained by the *deviance* analysis of the chromosome duplication rate were 51.32% for the F1 generation and 65.67% for the F2 generation (Figure 2A). In the chromosome duplication protocol analyses, the highest rate of duplicated plants was obtained using Protocol 2 (69.33%), whereas for Protocol 1, the rate was 51.32% (Figure 2A). The hybrids did not differ significantly between each other regarding the duplicated plant rate.

Dang et al. (2012) observed rates of duplicated plants ranging from 28% to 54%. Battistelli et al. (2013), in turn, obtained a higher percentage of duplicated plants, ranging from 59.1% to 80%, whereas the percentages of plants duplicated by Choe et al. (2012) varied from 5% to 57.1% in the evaluated hybrids.

The protocols used allow for some comparisons due to their divergent nature. Protocol 1 has a more practical methodology and is similar to that of Deimling, Rober and Geiger (1997), differing only in the colchicine concentration. Protocol 2, in turn, has a more laborious methodology because seven or more days are necessary for the plant to grow sufficiently for its roots to be subjected to the duplication solution. Moreover, after reaching the ideal size, the plants need to be removed from the trays and washed with water to remove the sand and vermiculite that adhere to the roots. This stage is laborious and requires caution to avoid the destruction of the roots before the duplication stage.

The protocols used were efficient for the duplication of the plants. However, one of the objectives in this study was to obtain duplicated haploids and, thus, obtain doubled haploid lines. Therefore, statistical analyses were conducted in haploid plants that were duplicated to evaluate the efficiency of the two chromosome duplication methodologies. In the analysis of the duplicated haploid number, within the total number of haploids identified by flow cytometry, there were significant differences in the protocols and also in the generations. The adjusted averages obtained by the *deviance* analysis of the doubled haploid rate using the total number of haploids identified by flow cytometry were 50.96% in generation F1 and 64.61% in generation F2 (Figure 2B).

Protocol 2 was more efficient than Protocol 1 in this analysis. For the haploid duplication rate, Protocol 2 presented an average of 65.94%, whereas Protocol 1 presented an average of 50.97% (Figure 2B). This finding indicates that despite having a more laborious methodology, Protocol 2 is more efficient for haploid duplication and attainment of doubled haploid lines. The hybrids used did not present significant differences regarding haploid duplication.

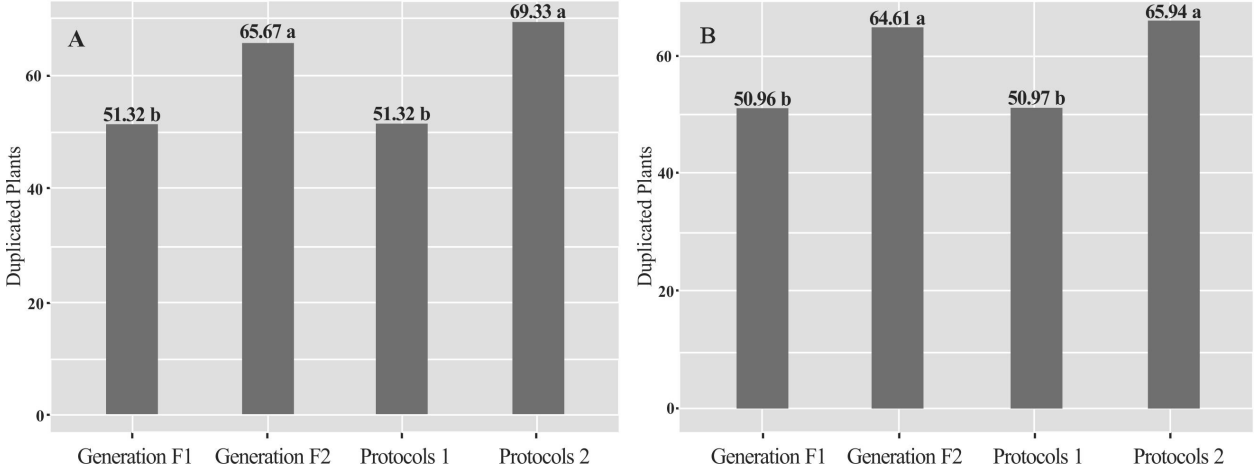

Figure 2: Mean percentages of the duplicated plants total (A) and duplicated haploids (B) considering generation F1 and F2 and protocols 1 and 2. Mean percentages followed by the same letter belong to the same cluster. The values are based on the Mahalanobis distance with a cutoff of 5% probability.

It is important to emphasize that the cytometry results indicated the success of the chromosome duplication in the somatic cells of the plant (leaf), which does not guarantee that it is fertile and will produce seeds. The fertility of the tassel can be estimated using to the percentage of plants that produce pollen and are self-fertilizing, which can be used to estimate the quantity of seeds harvested in duplicated haploid plants.

Percentages of self-fertilized duplicated plants

Companies seek to improve the attainment of doubled haploid seeds in their programs. Thus, the quantities of seeds harvested in each protocol and the number of doubled haploid seeds were determined.

Using the *deviance* analysis, we observed significant differences regarding the total number of duplicated plants that were self-fertilized for hybrids and the protocols.

For Protocol 2, an average of 65.19% of self-fertilized plants was observed, whereas in Protocol 1, the average was 30.89%. It was possible to verify that the efficiency of the duplication goes beyond the response of the hybrids to the colchicine, which was verified by the flow cytometry analysis. For Protocol 1, with average of 51.32% of duplicated plants (Figure 2A) determined by flow cytometry, a low average percentage of self-fertilized plants was found in the field (30.89%). For Protocol 2, high average percentages of duplicated (69.33%) (Figure 2A) and self-fertilized (65.19%) plants was observed. As observed by Battistelli et al. (2013), it is important to emphasize that the results of chromosome duplication by the flow cytometry technique are evaluated only in the somatic cells of the leaves. In the field, a lower vigor and delay in the development of the duplicated haploid plants was observed compared to plants that did not respond to the colchicine.

For the *deviance* analysis, significant differences were observed for the hybrids used. However, in the grouping based on the *Mahalanobis* distance, the hybrids did not differ in the averages. Despite that, there was a considerable difference between the hybrids, with averages of 16.06% for the GNS 3264 hybrid, 23.04% for the GNS 3264 hybrid, 23.04% for the DKB 393 hybrid, 30.89% for the GNS 3032 hybrid, and 32.16% for the GNS 3225 hybrid.

These differing values, even for plants that belong to the same group based on the *Mahalanobis* distance, presented practical differences when the field experiment was conducted. GNS 3264 hybrid plants, for example, showed less vigor and development, as well as a delay in the formation of the tassel and liberation of the style and stigma, compared to the other hybrids.

The analyses by flow cytometry allowed the tracking of duplicated haploid plants in the field, and thus, it was possible to analyze which plants had fertile tassels and were self-fertilized. In the *deviance* analyses, significant differences between the generations of hybrids were observed. The adjusted averages obtained by the *deviance* analysis of the self-fertilized double-haploid rate were of 16.04% in generation F1 and 9.72% in generation F2.

Quantities of seeds obtained by duplicated plants

The quantities of ears harvested in this experiment are shown in Table 2. It was observed that more ears were harvested using Protocol 2 than Protocol 1. These data were expected because Protocol 2 was superior to Protocol 1 in the statistical analyses of duplicated plant self-fertilization.

It is interesting to note that doubled haploid technology, although advantageous, offers a much smaller final balance of harvested seeds than the number of seeds used at the beginning of this study. From the crosses of the four hybrids with the haploid inducer line KEMS, 15314 seeds were harvested, from which only 1086 were selected by the R1-Navajo marker. From that total of selected seeds that were duplicated, only 537 survived in the field, and at the final stage, 82 ears were harvested.

Table 2: Quantities of ears harvested in the field and ears of doubled haploid plants after the self-fertilization of duplicated plants.

Hybrids	Quantity of harvested ears		Quantity of doubled haploid ears	
	Protocol 1	Protocol 2	Protocol 1	Protocol 2
GNS 3225	4	21	1	6
GNS 3032	4	14	2	5
GNS 3264	3	18	1	2
DKB 393	7	11	6	2
Total	18	64	10	15

The quantities of harvested ears presented in Table 2 originate from duplicated plants of varied ploidies. Thus, as the main objective of the technology is to obtain doubled haploid seeds, the number of ears of doubled haploid plants harvested was analyzed. From the total of 82 harvested ears, 25 were from doubled haploid lines (Table 2). Protocol 2, once again, was superior to Protocol 1 in this analysis, presenting a higher number of harvested doubled haploid ears. The hybrids varied in the number of harvested ears.

CONCLUSIONS

The hybrid type influenced the HIR. However, the hybrid type did not influence in the rate of chromosome duplication of the tested protocols.

The inducer line KEMS is effective in inducing haploids in a tropical climate and can be used as an inducer line in tropical maize breeding programs.

Other markers, in addition to the R1-Navajo system, should be used in the selection of haploid seeds.

Protocol 2 was more efficient for the chromosome duplication of haploids regarding the percentage of self-fertilized plants after duplication and for the attainment of doubled haploid lines.

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