Original Paper Open Access

Effectiveness of the *R1-navajo* embryo marker on sorting haploids in tropical maize germplasm

KF Milani[1,3], Alessandra G Baleroni[1,3*], HA Silva[1,3], AB Mendes-Bonato[2], RJB Pinto[1], CA Scapim[1]

[1]Agronomy Department, State University of Maringá, Paraná, Brazil
[2]Biotechnology, Genetics and Cell Biology Department, State University of Maringá, Paraná, Brazil
[3]These author contributed equally to the manuscript.
*Corresponding author: E-mail: le_gb@hotmail.com

Abstract

Modern maize breeding is currently based on Doubled Haploids (DH) technology. In following the steps to produce inbred lines by DH, sorting the haploids are the most important of them. Haploid inducers usually carry the *R1* gene, responsible of purple pigmentation on seeds. Navajo is a typical phenotyping from this gene and leads to purple coloration on endosperm and embryo of the diploids but any pigmentation on haploid kernels. Aimed to analyze the effectiveness of *R1-nj* on sorting true haploids over tropical sources, two different experiments were conducted. In the first experiment, kernels analyzed came from the following induction cross (L47 x IL47), (P9 x IP9), (2B707 x IL47), and (2B707 x IP9) in which IL47 and IP9 were the tropical haploid inducers. In the second experiment, KHI (Krasnodar Haploid Inducer) was the haploid inducer and three commercial were adopting as sources. Putative haploids were sorted by *R1-nj* expression and the haploid level was checked by chromosome counting our phenotyping at the flowering stage. Overall, very low true haploid discovery rates were observed. 2B707 was the most reliable source in displaying true haploids. The ratio of this effectiveness among the two experiments to *R1-nj* marker ranged from 8.80 to 30.2%. These results can be explained by the presence of inhibitory genes on the tropical sources. Additionally, at the flowering stage, haploids plants had data of spontaneous doubling collected and summarized. Recovery on the female organs seems to be higher than male. Haploids from the IAC125 source exhibited best fully spontaneous doubling among the sources analyzed.

Keywords: doubled haploids, bayesian Inference, *C1-I* gene, spontaneous doubling

Introduction

Doubled haploid (DH) technology is well established in the United States, Europe and China to support maize breeding, but its application to tropical areas is still limited (Chen et al, 2009; Dang et al, 2012; Batistelli et al, 2013; Melchinger et al, 2013; Chaikam et al, 2015). In this approach, which is nowadays the central focus of modern maize breeding, faster development of homozygous inbred lines are provided, carrying out advantages in maize breeding programs as reduced time to develop the parents and also testing the hybrids. Inbred lines are fast developed in this case because just two generations on the field are necessary to get the seeds from a DH line compared to six or more generations required by the common methodology, based on consecutive generations of selfing (Geiger and Gordillo, 2009; Dang et al, 2012). Besides increasing in selection gain as it only additive variance are involved on the selection, also facilities on introgression and transgene fixation are display as advantages (Röber et al, 2005; Dang et al, 2012; Batistelli et al, 2013).

Haploids from maize can be produced in vivo via induction crosses with a genotyping that has a genetic trait to generate a portion of the kernels where the embryos have half of the number of chromosomes from a diploid cell. Steps to develop DH lines were set out by Prigge and Melchinger (2012) and initially include an induction cross between a source germplasm, which provide the genetic variance to be explored, and the haploid induction. The next step is to sort the haploid kernels and then submit the haploids to chromosome doubling and finally selfing on the D0 plants to seed set production (Röber et al, 2005; Geiger and Gordillo, 2009). Among these steps, sorting of haploid seeds require a fast and accurate protocol (Melchinger et al, 2013; Chaikam et al, 2015).

Most of induction haploids carry the *R1-nj* which encodes anthocyanin pigmentation on kernels. The phenotyping characteristics from Navajo expression are purple coloration on crown of endosperm and scutellum of embryo of the diploids seeds (Nanda and Chase, 1966; Chaikam and Prasanna, 2012). Thereby, when gynogenetic haploids are generated in the induction crosses, triploid endosperms carry one *R1* allele from the inducer, same as the diploid kernels. On the other hand, the embryos, which do not carry chromosomes from the inducer genotype due the disruption fertilization, any *R1* are expected be present on the scutellum, so no purple pigmen-

 RECEIVED 9/01/2016

tation is identified. To promoting purple expression, some structural genes as *A1*, *A2*, *C1*, *C2*, *Bz1*, and *Bz2* and also an important regulatory gene named *C1* are required (Coe Jr, 1962; Geiger and Gordillo, 2009; Sharma et al, 2011; Chaikam et al, 2015). *C1* gene plays a regulatory role on the anthocyanin pathway in seeds, interacting with *A*'s, *Bz*'s, and *R1* genes (Cone et al, 1993).

The protein resulting from the *C2* gene makes a conversion of phenylpropanoid derivatives to chalcones. After some reactions, chalcones are metabolized in dihydroflavonol. The *A1* gene encodes the enzymes which convert dihydroflavonol to leucoanthocyanidin. Following the pathway, the *A2* gene encodes the protein responsible for transforming leucoanthocyanidin to anthocyanidins. The *Bz1* and *Bz2* genes perform the final step, converting anthocyanidin to anthocyanin (Hanson et al, 1996; Lesnick and Chandler, 1998; Sharma et al, 2011).

Several studies have shown that tropical maize germplasm could not carry the genes to the full pathway of anthocyanin biosynthesis, indicating the background effect of the source on the phenotype expression (Dang et al, 2012; Chaikam et al, 2015). Moreover, some inhibitor genes such as: *C1-1*, *C2-Idf*, and *in-1D* were described to suppress the purple pigmentation on embryos and endosperms. Mutant allele *C1-1* was considered to be the most important inhibitor and encodes a protein which competes with *C1* protein, the wild type, encoded by the activator site of anthocyanin, and works as suppressor of transcriptional on heterozygous *C1-I/C1* due the dominance of *C1-I* over the wild type *C1* (Paz-Ares et al,1990; Chaikam et al, 2015).

The *R1* gene has a dominant effect over the recessive, but its expression can vary due environmental conditions (Chaikam and Prasanna, 2012; Chaikam et al, 2015). As result of non-reliable sorting, many haploids selected were false positives. All the efforts could become the DH technology not applicable even over high Haploid Inducer Rate (HIR) when adopting tropical germplasm as source.

A brief summary of Brazilian experiments on the doubled haploids subject reveals an unexpected or undesired number of false haploids among the putative haploid kernels fraction, or even a large number of tetraploids inside the amount of doubling plants after colchicine treatment (Belicuas et al, 2007; Batistelli et al, 2013; Couto et al, 2013). In this way, our objectives were firstly determining the effectiveness of the *R1-nj* maker system on sorting true haploids in tropical maize sources. Secondly, we aimed to measure indirectly the HIR from the inducers adopted in this work, and finally evaluating the spontaneous doubling from three tropical hybrids used as sources on the induction crosses.

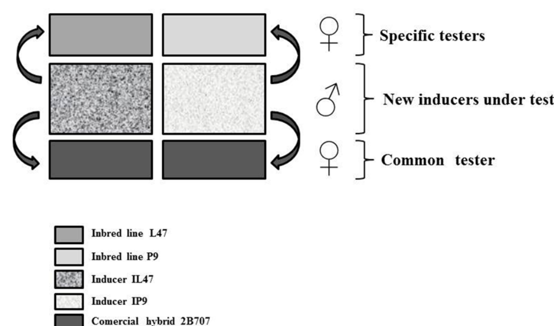

Figure 1 - Field scheme adopting on the inductions crosses.

Materials and Methods

Plant material, induction crosses and kernel sorting by R1-navajo expression

Two different experiments were conducted to evaluate the effectiveness of the *R1-nj* expression on sorting true haploid kernels. In the first experiment, two new inducer populations were developed from crosses between KHI (Krasnodar Haploid Inducer) and two tropical lines by the State University of Maringá. They are named as IL47 and IP9. Aiming analyze the effectiveness of true haploid sorting by this marker system, each new population haploid inducer were adopted as male in induction crosses (Figure 1). As testers (sources), the same tropical inbred lines used to develop each population were adopted as female (nominated as specific tester), beyond the commercial hybrid 2B707 (nominated as common tester).

After maturity, kernels from 10 ears from each induction cross were harvested, dried and sorted once more adopting the embryo marker coloration as criterion in putative haploids, F1's diploids or outcrossing. A total of 5062 kernels were analyzed from the induction cross L47 x IL47, 4439 from P9 x IP9, also 5062 from 2B707 x IL47 and 4439 from 2B707 x IP9. Later, the group of putative haploids sorted from each crossing had the chromosome number checked through chromosome counting from root tip cells to evaluate the effectiveness of *R1-nj* on sorting true haploids.

In the second experiment, three one-way commercial hybrids were used in induction crosses: P2530, P3989 and popcorn IAC125, KHI was the haploid inducer. Kernels were also sorted into putative haploids, diploids F1's and outcrossing. To the induction cross with P2530, 15734 kernels were generated, 14378 to P3989 and 2754 to IAC125.

Chromosome number count and phenotyping on the field

Putative haploid kernels from the first experiment had chromosomes counted to analyze the efficiency of *R1-navajo* marker in sorting the true haploids. For this purpose, seeds were germinated in petri dishes with germination paper at 25°C for 3 days. Roots tips

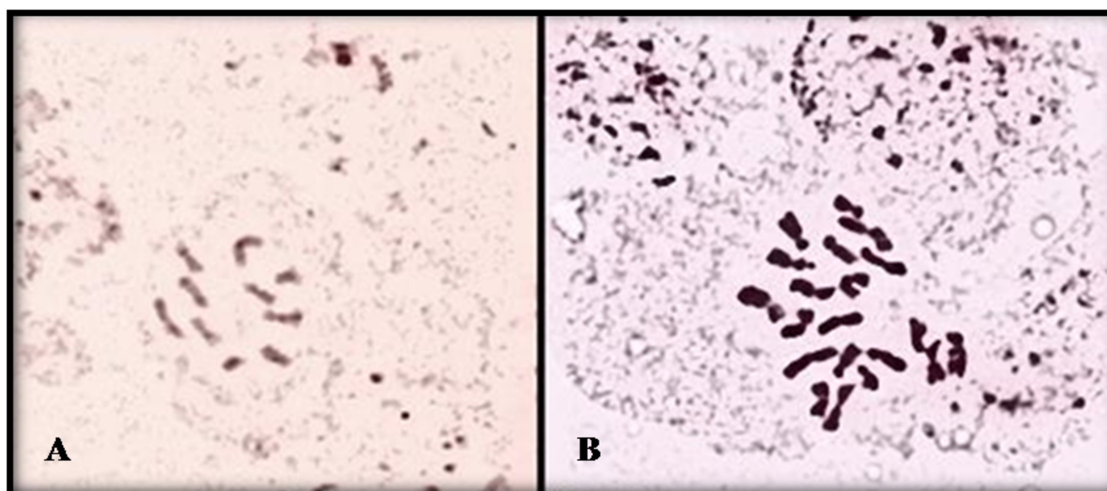

Figure 2 - Root tip cells. A: true haploid seedling (10 chromosomes). B: false positive (20 chromosomes).

were excised and immersed in a colchicine solution 0.05% for 2 hours and fixed in Carnov-metanol solution (3:1). After the procedure, the material was hydrolyzed in hydrochloric acid and the chromosome staining lacto-acetic orcein 1%. Glass slides were prepared adopting the smashing technique and then analyzed in an optic microscope, using immersion oil. Cells showing 10 chromosomes displayed the true haploids while were ones that had 20 chromosomes were classified as false positives (Figure 2).

To measuring the true and false haploids among the putative haploids from the second experiment, 100 seeds classified as putative haploids from each inducer crosses were sowed in a greenhouse and their phenotyping was analyzed in the flowering stage. Plants showing weakness, thin and straight leaves, low height, down vigor, foliar disease susceptibility, absence or incomplete development of the reproductive organs as tassel and ears or else, complete reproductive organs but poorly developed, defective or infertile were sorted as a true haploid plants. On the order hand, if the plant showed vigor and was well developed, it was classified as false haploid, highlighting the seed was a F1 cross between the source and the inducer, therefore from a diploid kernel.

Spontaneous doubling evaluation

Additionally, in the second experiment spontaneous doubling were evaluated. For this purpose, data of putative haploids which showed some fertile restore were collected as the number of plants which display some fertility as the presence/absence of ears, style-stigma, tassel and pollen were by source germplasm.

Statistics and data analysis

Statistical analysis was performed using Bayesian Inference. For these data, where the variable responses are dichotomous, the probability density function a posteriori is Yi~Bernoulli (θ) and:

$$t = \sum_{i=1}^{n} Y_i \quad Binomial(n,\theta)$$

Bernoulli or Binomial is used to model situations where each trial has one of two possible outcomes, for example: putative haploid and diploid or true haploid and false haploid. In the Binomial model, θ is a ratio and means the kernel probability to be sorted into putative haploids or true haploids from a sample which has n size and the where t is the number of kernels sorted as putative haploids or true haploids, respectively. Probability distribution of t is a Binomial with the parameters n e θ as showing in the model below:

$$P(T = t \mid \theta) \;=\; \binom{n}{t}\theta^{t}(1-\theta)^{n-t},\; t \;=\; 0.1,\; \ldots,\; n$$

Probability density function *a posteriori* conjugate to the Binomial distribution chosen to θ is Beta with the parameters α and β, following:

$$\pi(\theta) = \frac{1}{beta(\alpha,\beta)}\theta^{\alpha-1}(1-\theta)^{\beta-1}$$

No informative *priories* were implemented as α, β ~ N (0, 10 - 6) and the marginal distributions were estimated on the program «OpenBugs» (Rossi, 2011). For parameter estimates, 10,000 values were generated by the MCMC (Monte Carlo Markov Chain) in an interactive process, from these, 1000 initial values were burned. Convergence was analyzed in the package "CODA" from R and checked by Heidelberger and Welch (1983) and Geweke (1992) tests (R Development Core Team, 2014).

For the first experiment, all the factors (sources and inducers) were analyzed to generate the best model. Models were compared using the Bayesian DIC (Deviance Information Criterion) when the small DIC were considered as the best adjusted (Spiegelhalter et al, 2002). Contrasts were built to compare the estimate ratio and parameters and were considered significant at 95% confidence if the confidence interval does not include the value zero.

Table 1 - Estimated parameters to ratio and confidence interval and contrasts to putative haploids ratio among sources for the first and second experiment.

	Sources	Estimated parameters		
		$p_{2.5\%}$	ratio	$p_{97.5\%}$
1° Experiment				
	Inbred line L47	1.47	1.82	2.20
	Inbred line P9	14.67	15.77	16.91
	Hybrid 2B707	0.60	0.84	1.11
	Hybrid 2B707	0.62	0.84	1.09
		Contrasts		
	L47 vs. P9*	-15.13	-13.96	-12.79
	L47 vs. 2B707*	0.53	0.98	1.44
	L47 vs. 2B707*	0.54	0.98	1.42
	P9 vs. 2B707*	13.80	14.93	16.10
	P9 vs. 2B707*	13.80	14.93	16.08
	2B707 vs. 2B707	-0.34	0.00	0.34
2° Experiment				
	P2530	12.58	15.22	18.04
	IAC125	11.11	13.45	15.98
	P3989	9.54	11.55	13.75
		Contrasts		
	P2530 vs. IAC125	-1.85	1.76	5.48
	P2530 vs. P3989*	0.28	3.66	7.18
	IAC125 vs. P3989	-1.246	1.90	5.12

* Contrasts significantly different with 95% of confidence.

Results

Putative haploids ratio

In the first experiment, it was found that the highest putative haploids ratio occurred when the source was the popcorn P9 inbred line, followed by L47 and the commercial hybrid 2B707, respectively. The estimated ratio was 15.77%, with confidence interval [14.67, 16.91] for source P9. Inbred line L47 had an estimated ratio of 1.82% [1.47, 2.20] of putative haploids and 0.84% [0.60, 1.11] for the 2B707 hybrid (Table 1). In this experiment, common endosperm maize had a smaller putative haploids ratio than popcorn source. Common tester 2B707, which has a flint endosperm, sorted less number of seed as putative haploid among all sources. No effect of the inducer factor as detected on the first experiment (data not shown).

In the second experiment, the putative haploids ratios were more similar among the sources. For the popcorn source IAC125, estimated ratio was 13.45% [11.11, 15.98]. Common hybrid sources showed 15.22% [12.58, 18.04] for P2530 and 11.55% [9.54, 13.75] for P3989. The IAC125 vs. P2530 and P3989 contrast did not display significant difference at 95% confidence in putative haploids ratio. However, the contrast P2530 vs. P3989 showed P2530 produced more putative haploid seeds than when P3989 was used as the source.

True haploids discovery ratio (R1-nj effectiveness)

True haploids discovery ratio in the first experiment highlighted hybrid 2B707 as the most effective in ranking true haploids among the sources evaluate. Estimated ratio for this hybrid ranged from 22.45% [12.02, 35.13] to 31.67% [18.33, 46.82]. The second best tester in sorting true haploids was the dent inbred line L-47 with ratio of 15.95% [8.37, 25.23], following by the popcorn inbred line P9 with 8.80% [6.52, 11.33], which was worst reliably genotyping in the first experiment (Table 2).

In the second experiment, once more, popcorn was the worse on sorting real haploids using *R1-nj* as tool. True haploids among the putative haploids coming from the induction crosses with IAC125 performed a ratio of 3.02% [0.64, 7.18]. The contrast P2530 vs. P3989, both with flint endosperm, does not display significant difference on true discovery ratio. To P2530 source, the true discovery ratio was 16.68% [9.78, 25.22] and P3989 had a similar ratio of 15.04% [8.59, 23.08].

Considering the true haploids discovery ratio and the total number of seeds produced by each induction cross, it was possible to indirectly get an inference about HIR from these two haploid inducers. In the first experiment, a HIR of 0.22% to IP9 and 0.54% to IL47 were estimated (data not shown). The ratio of HIR to KHI on the second experiment was 1.96% in crossing with P2530, 1.49% with P3989 and 0.26% with IAC125 source. Nevertheless, as expressed by Dang et al (2012), when the germplasm carries inhibition genes of anthocyanin pathway, some haploids can be wrong sorted on diploid fraction. In their work, the ratio of haploid kernels with any purple expression ranged from 0 to 10.2%. Similar HIR was detected in Brazil by Couto et al (2013) and Batistelli et al (2013) adopting the inducers W23 and KEMS.

Table 2 - Estimated parameters to ratio, confidence interval and contrasts to the true haploids discovery ratio among sources for experiment 1 and 2.

	Sources	Estimated parameters		
		$p_{2.5\%}$	ratio	$p_{97.5\%}$
1° Experiment				
	Inbred line L47	8.37	15.95	25.23
	Inbred line P9	6.52	8.80	11.33
	Hybrid 2B707	18.33	31.67	46.82
	Hybrid 2B707	12.02	22.45	35.13
		Contrasts		
	L47 vs. P9	-0.79	7.15	16.84
	L47 vs. 2B707	-32.87	-15.72	0.43
	L47 vs. 2B707	-21.16	-6.50	7.55
	P9 vs. 2B707*	-37.94	-22.87	-9.43
	P9 vs. 2B707*	-26.51	-13.64	-2.99
	2B707 vs. 2B707	-8.78	9.23	27.43
2° Experiment				
	P2530	9.78	16.68	25.22
	IAC125	0.64	3.02	7.18
	P3989	8.59	15.04	23.08
		Contrasts		
	P2530 vs. IAC125*	5.78	13.66	22.55
	P2530 vs. P3989	-8.86	1.64	12.15
	IAC125 vs. P3989*	-20.65	-12.03	-4.40

* Contrasts significantly different with 95% of confidence.

Spontaneous doubling

Data from plants with some fertility restoration had different performance depending on the haploid sources. Although IAC125 had the smaller HIR and true haploids discovery ratios, haploids from this source had the highest restoration to ears, style-stigma and tassel, but pollen production was not detected in these plants (Table 3). Source P2530 showed an intermediate number of spontaneous doubling but was the only type which displayed pollen production while P3989 source had the worst performance of spontaneous doubling plants among the sources analyzed.

Discussion

Putative haploids ratios observed in both experiments are similar to those described in the literature. Couto et al (2013) found 27.78% of putative haploid rates by *R1-nj* sorting. In their work, the inbred line W23 was adopted as haploid inducer under tropical conditions in an induction cross with a flint hybrid. Batistelli et al (2013), detected a putative haploid rate ranging from de 0 to 10% following *R1-nj* expression and using the same haploid inducer. To KEMS haploid inducer, a syntactical variety, putative haploid rates ranging from de 1.9 to 32.1%. The putative haploid rates found by Dang et al (2012) ranged from 6.6 to 42.4%, studying three different haploid inducers.

On the true haploids discovery ratio, low efficiency of the *R1-nj* marker on tropical sources also was described by Belicuas et al (2007) and their pioneering research on DH subject in Brazil. These authors found 4 true haploids from 425 putative haploid seeds by SSR markers, performing 0.96% of true haploids discovery ratio, using the inducer W23 on induction crosses. Overall, independent of the induction crosses, a higher false positive number was discovered by the embryo marker expression in our two experiments, showing the low reliability of this marker system on tropical sources. Dang et al (2012) detected a rate among 3.4 to 15.7% of real haploids on the group sorting as putative haploids when it was checking by flow cytometry.

Chaikam et al (2015) studied a higher number of genotyping and verified non differences over color inhibition between flint and dent genotyping, although flint had displayed less color intensity. This factor can influence wrong sorting, especially on popcorn seeds which combine many complicated characteristics to visual score (small size of kernel, little embryo and non-flat surface of pericarp in the scutellum region). Popcorn kernels have smaller size also a little scutellum that becomes more complicated embryo viewing. These facts could explain the higher number of misclassifications in popcorn sources, but also the presence of some inhibition factors on the genotyping background.

Some studies had attributed more haploid misclassifications to flint than dent endosperms (Coe Jr, 1994) but, in most of these cases, just a few numbers of genotyping were compared. Röber et al (2005), highlighted best pigmentation on dent genotypes, in agreement with Chaikam et al (2015) whom described stronger pigmentation on dent lines.

In their recent work, Chaikam et al (2015) detected high suppression of the Navajo expression in trop-

Table 3 - Haploids displaying spontaneous doubling by reproductive structure.

Hybrid	Putative haploids	Ear	Style-stigma	Tassel	Pollen
	1	-	-	+	-
	2	+	+	+	-
	3	-	-	+	+
	4	-	-	+	-
	5	+	-	+	-
	6	+	-	+	-
P2530	7	-	-	+	-
	8	-	-	+	-
	9	+	+	+	-
	10	+	+	+	-
	11	+	-	+	+
	12	+	-	-	-
	13	-	-	-	-
	14	-	-	-	-
IAC125	1	+	+	+	-
	2	+	-	+	-
P3989	1	-	-	+	-
	2	+	-	-	-
	3	-	-	-	-
	4	+	-	-	-
	5	-	-	-	-
	6	-	-	-	-
	7	-	-	-	-
	8	+	-	+	-
	9	+	-	-	-
	10	-	-	-	-
	11	+	+	+	-
	12	+	-	+	-
	13	-	-	-	-

(+): presence (-):absence

ical germplasms. Analyzing the inbred lines group by adapting conditions (lowland tropical, subtropical and highland) they found differential influence of the adapting conditions over suppression anthocyanin pigmentation. The same authors evaluated several induction crosses with tropical sources including inbred lines, landraces and breeding populations and found dominant mutants to *C1* locus as the genetic factor to suppress anthocyanin biosynthesis. The mutant *C1-I* was detected as the most important responsible to inhibit purple coloration on endosperm and embryos at heterozygotes (*C1-I/C1*), leading to misclassification of the seeds as haploids (Paz-Ares et al, 1990; Chaikam et al, 2015).

Inhibition of purple coloration on kernels is more affected by broad genetic basis and adaptation of the genotypes. As concluded by Chaikam et al (2015), as broader as is the genetic base, less suppression of Navajo is detected. The source 2B707, a flint germplasm, was noted to have less misclassification of true haploids (Table 2) among all the sources, following by another flint source. The reliability of this source must be connected to the small frequency of the mutant alleles to inhibition or either to stability of this genotyping to *R1-nj* gene penetrance and anthocyanin expressivity. Eder and Chalyk (2002) also detected best results attributed to flint endosperm.

The misclassifications that arise in sorting haploids by *R1-nj* expression could be minimized if marker assisted selection is applied to identify the presence of inhibitory genes on maize breeding populations, at least to *C1-I* locus, especially to tropical and subtropical sources (Chaikam et al, 2015). Another possibility would be the introgression of genes like *Pl1* and *B1*, which are responsible for purple expression on the coleoptile and seedling roots, in the inducer genotyping as auxiliary tool to discard false positives after seed germination (Röber et al, 2005; Rotarenco et al, 2010).

Rotarenco et al (2007) suggested and Melchinger et al (2013) demonstrated a reliable and applicable way to sort haploids by adopting an inducer with high oil content. Development of high oil content haploid inducers adapted to tropical conditions in replacement of inducers based on *R1-nj* marker is an interesting outcome.

New insights into seedling treatment with exogenous anthocyanin pathway activators overcoming the inactivation by *C1-I* mutants also could be promising in DH research in order to promote more effective true haploid sorting.

It is important to highlight that the putative haploid rate never can be used as HIR synonymous because of overestimation. We can observe that less

than 30% of the seeds sorted as putative haploids were confirmed as real haploids. Methodologies to evaluate haploid inducer rate are described by Neuffer et al (1997) and Prigge et al (2012) and are based on morphological characteristics controlled by recessive genes as liguleless or glossy, expressed on young leaves. To solve the problem with the haploid misclassifications by the embryo marker in the overestimation of the haploid induction rate, Kebede et al (2011) suggest using misclassification rate to correct HIR when any of these morphological markers are available. Misclassification rate should be recorded through the phenotyping a portion among the seeds from all induction cross.

Over the spontaneous doubling in maize, it is usually lower than 20%. However, this trait is genotyping dependent and some sources could provide higher rates of doubling plants (Chang and Coe Jr, 2009). Even though, breeding DH lines from spontaneous doubling are not economically viable, some lines could be developed in the case of spontaneous doubling where the fertility is restored. However, in the second experiment, any fully fertile doubled plant was detected.

In our study, restoring was more pronounced on ears and tassel than skills and pollen. Some authors had described that female organs generally have higher fertility recovery rates and these rates can range from 25 to 96% (Han et al, 2006; Chang and Coe Jr, 2009). Male organs usually show less fertility. In the literature, male inflorescences that produce normal pollen grains are smaller than 46% in cases of spontaneous doubling (Liu and Song, 2000).The main cause of spontaneous doubling could be explain by phenomenon such as somatic cell fusion, endoreduplication, endomitosis or even other mechanisms not yet described (Liu and Song, 2000; Testillano et al, 2004).

Acknowledgements

The authors acknowledge the Brazilian research-funding agencies CAPES and CNPq for the financial support to this research.

References

Batistelli GM, Von Pinho RG, Justus A, Couto, EGO, Balestre M, 2013. Production and identification of doubled haploids in tropical maize. Genet Mol Res 12: 4230-4242

Belicuas PR, Guimarães CT, Paiva LV, Duarte JM, Maluf WR, Paiva E, 2007. Androgenetic haploids and SSR markers as tools for the development of tropical maize hybrids. Euphytica 156: 95-102

Chaikam V, Prasanna BM, 2012. Maternal haploid detection using anthocyanin markers, pp. 20-23. In: Doubled haploid technology in maize breeding: theory and practice. Prasanna BM, Chaikam V, Mahuku G eds. International Maize and Wheat Improvement Center, México

Chaikam V, Nair SK, Babu R, Martinez L, Tejomurtula J, Boddupalli PM, 2015. Analysis of effectiveness of R1-nj anthocyanin marker for in vivo haploid identification in maize and molecular markers for predicting the inhibition of R1-nj expression. Theor Appl Genet 128: 159-171

Chang MT, Coe Jr EH, 2009. Doubled haploids, pp. 127-142. In: Molecular Genetic Approaches to Maize Improvement. Kriz AL, Larkins A eds. Springer, Berlin

Chen S, Li L, Li H, 2009. Maize doubled haploid breeding [in Chinese]. China Agricultural University Press, Beijing

Coe Jr EH, 1962. Spontaneous mutation of the aleurone color inhibitor in maize. Genetics 47: 779-783.

Coe Jr EH, 1994. Anthocyanin genetics, pp. 279-281. In: The maize handbook. Freeling M, Walbot V eds. Springer, New York

Cone KC, Cocciolone SM, Burr FA, Burr B, 1993. Maize anthocyanin regulatory gene *pl* is a duplicate of *c1* that functions in the plant. Plant Cell 5: 1795-1805

Couto EGO, Chamma LMD, Bustamante FO, Von Pinho RG, Silva TN, 2013. Identification of haploid maize by flow cytometry, morphological and molecular markers. Ciênc Agrotec 37: 25-31

Dang NC, Munsch M, Aulinger I, Renlai, W, Stamp P, 2012. Inducer line generated double haploid seeds for combined waxy and opaque 2 grain quality in subtropical maize (*Zea mays* L.). Euphytica 183: 153-160

Geiger HH, Gordillo GA, 2009. Doubled haploids in hybrid maize breeding. Maydica 54: 485-489

Geweke J, 1992. Evaluating the accuracy of sampling-based approaches to the calculation of posterior moments (with discussion), pp. 169-193. In: Bayesian statistics 4. Bernardo JM, Berger JO, Dawid AP, Smith AFM eds. Oxford University Press, Oxford

Han X, Tang Q, Cao M, Rong T, 2006. Study on identifying methods of maize haploids induced by Stock 6. J Maize Sci 14: 64-66

Hanson MA, Gaut BS, Stec AO, Fuerstenberg SI, Goodman MM, Coe EH, Doebley JF, 1996. Evolution of anthocyanin biosynthesis in maize kernels: the role of regulatory and enzymatic loci. Genetics 143: 1395-1407

Heidelberger P, Welch P, 1983. Simulation run length control in the presence of an initial transient. Oper Res 31: 1109-1144

Kebede AZ, Dhillon BS, Schipprack W, Araus JL, Bänziger M, Semagn K, Alvarado G, Melchinger AE, 2011. Effect of source germplasm and season on the in vivo haploid induction rate in tropical maize. Euphytica 180: 219-226

Lesnick ML, Chandler VL, 1998. Activation of the maize anthocyanin gene *a2* is mediated by an ele-

ment conserved in many anthocyanin promoters. Plant Physiol 117: 437-445

Liu ZZ, Song TM, 2000. Fertility spontaneously restoring of inflorescence and chromosome doubling by chemical treatment in maize haploid. Acta Agron Sin 26: 947-952

Melchinger AE, Schipprack W, Würschum T, 2013. Rapid and accurate identification of *in vivo*-induced haploid seeds based on oil content in maize. Sci Rep 3: 2129.

Nanda DK, Chase SS, 1966. An embryo marker for detecting monoploids of maize (*Zea mays* L.). Crop Sci 6: 213-215.

Neuffer MG, Coe Jr EH, Wessler AR, 1997. Mutants of maize. CSHL Press, New York

Paz-Ares J, Ghosal D, Saedler H, 1990. Molecular analysis of the *C1-I* allele from *Zea mays*: a dominant mutant of the regulatory C1 locus. Embo J 9: 315-321

Prigge V, Xu X, Li L, Babu R, Chen S, Atlin GN, Melchinger AE, 2012. New insights into the genetics of in vivo induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics 190: 781-793

R Development Core Team, 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R-project.org. Accessed April 15, 2016

Röber FK, Gordillo GA, Geiger HH, 2005. *In vivo* haploid induction in maize performance of new inducers and significance of doubled haploid lines in hybrid breeding. Maydica 50: 275-283

Rossi RM, 2011. Introdução aos métodos bayesianos na análise de dados zootécnicos com uso do WinBUGS e R. 1st edn. Eduem, Maringá

Rotarenco V, Kirtoca IH, Jacota AG, 2007. The possibility of identifying kernels with haploid embryos using oil content. Maize Genet Coop Newsl 81: 11

Rotarenco VA, Dicu G, State D, Fuia S, 2010. New inducers of maternal haploids in maize. Maize Genet Coop Newsl 84:15.

Sharma M, Cortes-Cruz M, Ahern KR, McMullen M, Brutnell TP, Chopra S, 2011. Identification of the *Pr1* gene product completes the anthocyanin biosynthesis pathway of maize. Genetics 188: 69-79

Spiegelhalter DJ, Best NG, Carlin BP, Van der Linde A, 2002. Bayesian measures of model complexity and fit (with discussion). J R Stat Soc 64: 583-639

Testillano P, Georgiev S, Mogensen HL, Dumas C, Risueno MC, Matthys-Rochon E, 2004. Spontaneous chromosome doubling results from nuclear fusion during in vitro maize induced microspore embryogenesis. Chromosoma 112: 342-349