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ARTICLE

Identification of haploids and diploids in maize using seedling traits and flow cytometry

Alessandra Guedes Baleroni[1], Fabrícia Ré[1], Andressa Pelozo[1], Samuel Henrique Kamphorst[2*], José Walter Pedroza Carneiro[1], Robson Marcelo Rossi[1] and Carlos Alberto Scapim[1]

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Abstract: *The seedling traits is a simple and non-destructive methodology used to identify haploids/diploids in maize. This study aimed at establishing an optimal germination temperature to evaluate this methodology and verify its effectiveness. Haploid and diploid seeds, obtained from crosses with the haploidy inducer TAIL9, were classified using the R1-Navajo marker and placed to germinated in growth chambers maintained at different temperatures. After 96 h, radicle and coleoptile lengths and the number of lateral seminal roots were determined. Cut-off points for these traits were established using Receiver Operating Characteristic curves and a new haploid/diploid classification was performed and compared to the plant phenotype (gold standard) to obtain false discovery rates and false negative rates. The seedling traits methodology successfully differentiated haploids and diploids, proving to be effective in eliminating false positives, selected by the R1-Navajo marker. The temperature of 30 °C was established as the optimal germination temperature for this study.*

Keywords*: Doubled haploid, R1-Navajo, haploid inducer*

INTRODUCTION

Maize inbred lines can be obtained through the conventional method or the use of doubled haploid (DH) technology in breeding programs (Ribeiro et al. 2018). The conventional method is expensive and requires six to eight generations of self-fertilization of the inbreeding families to achieve the desired level of homozygosity. Compared to the conventional methodology, the main advantage of the DH technique is the shorter time required to obtain inbred lines (Battistelli et al. 2013), which is reduced to at least 18 months until full inbreeding is achieved. Additionally, the DH technique guarantees genetic purity of the inbred lines because all seeds generated from a single haploid seed will be genetically identical.

For the large-scale production of DH inbred lines, the precise and efficient separation of haploid from diploid seeds after the induction of haploidy is one of the most critical requirements of this method and must be accomplished before haploid seeds are exposed to treatment for chromosomal duplication (Chaikam et al. 2016). Seed classification is often conducted via the *R1-Navajo* marker, which is the result of the expression of the *R1-nj* gene present in the genome of inducers (Chase 1952). This dominant gene provides pigmentation of the endosperm and embryo of the diploid seeds. In the case of gynogenetic

***Corresponding author:**
E-mail: samuelkampho@hotmail.com
ORCID: 0000-0001-9727-3539

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[1] Universidade Estadual de Maringá (UEM), Av. Colombo, 5790, 87.020-900, Maringá, PR, Brazil
[2] Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Av. Alberto Lamego, 2000, Parque Califórnia, 28.013-602, Campos dos Goytacazes, RJ, Brazil

haploids, only the endosperm is anthocyanin-pigmented because the inducer DNA is not inherited by the embryo. Thus, the haploid seeds have purple endosperm, and the embryo remains white (Nanda and Chase 1966, Prigge et al. 2011).

Although the *R1-Navajo* marker is practical for the visual separation of haploids and diploids, it has a low reliability because it can be totally or partially inhibited in induction crosses, especially in tropical genotypes. In these genotypes, the occurrence of genes that inhibit the anthocyanin production pathway, such as *C*1-I (Chaikam et al. 2015), is frequent. Additionally, false positives (Prigge et al. 2011, Chaikam et al. 2016, Silva et al. 2020) and false negatives (Chaikam et al. 2016) can be common during visual separation. Thus, obtaining DH inbred lines becomes costly, because numerous diploid seeds move to the chromosomal duplication stage. Thus, the use of non-destructive methods, allowing the early elimination of diploid seeds, may improve the efficiency in obtaining DH (Choe et al. 2012).

Another tool used in the identification of haploids is based on the seedling traits, such as radicle length, coleoptile length, and the number of seminal roots. It is a simple, non-destructive, reliable, and effective procedure for the early identification of haploids in maize. Chaikam et al. (2017) studied these traits in seedlings germinated at 28-30 °C and were able to distinguish haploids from diploids with complete inhibition of the *R1-Navajo* marker.

Seed germination is influenced by the genotype and the environment. Water, oxygen, and favorable temperatures (T) are essential for good germination. For each crop, there are maximum, minimum, and optimum T for germination. In environments with temperatures above the maximum or below the minimum, seeds usually do not germinate. According to Cochrane et al. (2014), seeds may vary in their environmental responses, depending on the range of the germination T. Therefore, establishing the optimum T ensures good germination percentage and speed (Nascimento 2013).

Some studies evaluating alternative haploid selection methods have used the ROC (Receiver Operating Characteristic) curve to establish cut-off points and evaluate the discriminatory capacity of a diagnostic test compared with the standard (*gold standard*) test. Silva et al. (2020) used this analysis when studying the length of stomatal guard cells. In this context, the goal of this study was to determine the optimal T for germination to employ the methodology of seedling traits and verify whether this methodology is efficient in separating haploid and diploid individuals to eliminate the false positives selected by *R1-Navajo*.

MATERIAL AND METHODS

Haploidy induction crosses

Three cultivars (AG4051, CD384, and IAC Nelore) were subjected to haploidy induction by crosses with the TAIL9 inducer. The crosses were conducted in a pollination field, in the 2017/18 crop, when the inducer was used as pollen donor and the cultivars were the female genitors. TAIL9 is a tropical inducer developed in Mexico by the International Maize and Wheat Improvement Center (CIMMYT) in conjunction with the University of Hohenheim in Germany. This inducer contains the *R1-nj* gene, which promotes purple pigmentation in maize grains, haploid induction rate higher than 8%, high pollen production and desirable agronomic traits for cropping maize under tropical Brazilian climate conditions (Prasanna et al. 2012).

Classification of the seeds through R1-Navajo

The ears from the crosses were harvested at physiological maturity, individually threshed, and dried to 14% moisture. The seeds were then visually classified by the color of the endosperm, embryo and stored in a cold chamber (Bewley and Black, USA). Thus, according to *R1-Navajo*'s expression, seeds with purple coloration in the endosperm and a white embryo (no purple coloration) were identified as possible haploids, whereas seeds with purple endosperm and embryo were considered diploids (Nanda and Chase 1966).

Evaluation of haploid and diploid seedling traits

The experimental design used was a completely randomized with the treatments in a 3x2x4 factorial scheme (cultivar x ploidy x temperature), with 20 replications. Thus, we randomly selected 20 seeds of the fraction classified as haploid and 20 diploid seeds from each cross (AG4051 × TAIL9, CD384 × TAIL9, and IAC Nelore × TAIL9). These seeds were positioned on moist germination paper placed under 28 × 38 cm glass sheets. The plates, each containing 10

seeds, were packed in trays with water, placed in growth chambers, and maintained at 20, 25, 30, or 35 ºC. Each seed was considered a replication for the later confirmation of ploidy by flow cytometry. After 96 h, the number of lateral seminal roots (NLSR) and the lengths of the radicle (RL) and coleoptile (CL) were determined in all seeds from the visible emergence point to their respective tips.

Transplantation and confirmation of the phenotype

After the measurements, the seedlings were transplanted into 11-L pots in a greenhouse, with two seedlings per pot. After 45 d, the phenotype of each surviving plant was confirmed, based on traits such as plant vigor, upright leaf, and color, which could distinguish haploids from diploids. Haploids, when compared to diploids, usually have less vigor and erect, narrow, pale leaves (Melchinger et al. 2013).

Evaluation of plant ploidy by flow cytometry

To assess the ploidy of plants and compare it with that of the previously analyzed phenotypes, we performed flow cytometry (Galbraith et al. 1983) at 15 to 20 d after seedling transplantation. Only seeds previously selected as possible haploids by *R1-Navajo* and that survived transplantation were used. Samples (20-30 mg) of young leaves from genitors and the descendants were used. The samples were fragmented with a scalpel into a Petri dish and kept on ice containing 1 mL of cold LB01 buffer to facilitate the release of the nuclei (Doležel 1997).

The suspended nuclei were aspirated with a plastic pipette covered by two layers of gauze at the tip to prevent the collection of leaf fragments. In the next step, the nuclei suspension was filtered through nylon mesh (50 µm) and transferred to a 5-mL polyethylene tube. Before being analyzed in the BD FACSCalibur flow cytometer (Becton Dickinson, San Jose California, USA), the nuclei suspension was stained with 25 µL propidium iodide (1 mg mL^{-1}), and 5 µL of RNase were added to each sample (Doležel et al. 1992). In total, 10.000 nuclei from each sample were observed. The obtained histograms were analyzed using the Cell Quest software (BD CellQuest 2000). The level of ploidy was determined by comparing the position of the G1 peak of each sample with that of the female parent (standard) G1 peak of known ploidy (diploid).

Statistical analysis

The data were analyzed using generalized linear models (GLM) (Nelder and Wedderburn 1972). The Poisson distribution (Poisson 1837) is suitable to fit data set that does not follow the normal distribution, and dependent variables have only positive numbers without upper limit, such as count data. Thus, for the NLSR, the Poisson distribution with the *log-link* function was used, as presented below. For the radicle and coleoptile lengths, the same model was adopted using the normal distribution, thus adding the variance parameter. The model adopted was:

$$Y_{ijk} \sim \text{Poisson}\ (\mu_{ikj})\ ;\ log(\mu_{ikj}) = \mu + C_i + P_j + T_k + CP_{ij} + CT_{ik} + PT_{jk} + CPT_{ijk},$$

where Y_{ijk} is the NLSR observed for cultivar *i,* ploidy *j,* and T_k; μ is the overall mean; C_i is the effect of the cultivar; P_j is the effect of ploidy; T_k is the effect of *T*; CP_{ij} is the effect of the interaction between the cultivar *i* and ploidy *j*; CT_{ik} is the effect of the interaction between the cultivar *i* and T_k; TPj_k is the effect of the interaction between ploidy *j* and T_k; and CPT_{ijk} is the effect of the interaction between the cultivar *i*, ploidy *j,* and T_k.

A type III *deviance* analysis was performed according to the model used for each response variable, evaluating the significance of isolated factors and their interactions. The differences between ploidies were classified based on the χ^2 (chi-square) test (Cordeiro and Demétrio 2011) for *deviance* analysis at 5% significance level because this factor only has two levels. For the other significant factors and interactions, the contrasts between means were determined and the Wald test (Wald 1943) was carried out at 5% significance level. All analyses were performed in the SAS statistical program using *proc genmod* (SAS Institute Inc 2017).

To verify the application of the seedling traits as a methodology for haploid identification among diploid seeds, the plant phenotype was considered the *gold standard* for the ploidy level, where the more vigorous plants were classified as diploids. To establish the optimum germination T, the *ROCR* and *Epi* packages of the *R* program (R Core Team 2018) were used to generate the ROC curves and establish individual cut-off points (*thresholds*) for the analyzed variables for each cross (cultivar × inducer). Subsequently, seedlings of equal or greater value than the established cut-off point were

classified as diploids and those with lower values than the cut-off point were classified as haploids. This new classification was the basis for creating contingency tables containing the true positive values (TP - diploids identified as diploids), true negatives (TN - haploids identified as haploids), false positives (FP - haploids identified as diploids), and false negatives (FN - diploids identified as haploids). Then, the accuracy $\left(\frac{TP + TN}{total}\right)$ and the false discovery rates $\left(FDR = \frac{FP}{FP + TP}\right)$ and false negative rates $\left(FNR = \frac{FN}{FN + TP}\right)$ were calculated.

The quality of the rating based on the established cut-off points was assessed through the area under the curve (AUC), obtained using the ROC curve. The agreement and association between the *gold standard* and the diagnostic test (seedling traits) were determined using the coefficient of Kappa (Cohen 1960) (H_0: no agreement between the tests) and the McNemar tests (McNemar 1947) (H_0: measures are similar), respectively. Subsequently, the coincidence between the phenotypic classification of possible haploids, previously selected by *R1-Navajo*, and the results of flow cytometry were investigated using Genes software (Cruz 2013).

RESULTS AND DISCUSSION

Determination of the optimum germination temperature

The *deviance* analysis showed significant effects of the main factors cultivar (C), ploidy (P), and temperature (T) on the three response variables. Regarding the interactions, P × T and C × P × T for radicle length (RL), and C × T and P × T for coleoptile length (CL) and for the number of lateral seminal roots (NLSR) were statistically significant (Table 1). Similar to the results of Chaikam et al. (2017), we observed significant differences between haploid and diploid seeds for RL, CL, and NLSR. Regardless of cultivars and T, haploid seeds had shorter RL and CL and a lower NLSR compared with those of diploids (Table 1 and Figure 1).

T is considered one of the most important factors in the germination of seeds (Nerson 2007) because it can interfere with water absorption and compromise the development of seedlings. T of 30 °C promoted greater radicle and coleoptile growth and more seminal roots, regardless of other factors (Table 1).

At T of 25 and 30 °C, there was a significant difference between the ploidies in RL and between cultivars and ploidies in CL and NLSR. At 35 °C, there were differences between cultivars in CL and between ploidies in NLSR. At 20 °C, we observed differences between ploidies in RL and between cultivars and ploidies in NLSR (data not shown). Thus, the T

Table 1. Summary of type III *deviance* analysis and comparison of means between the main effects for three traits

Sources	df	Radicle length (RL)		Coleoptile length (CL)		Number of lateral seminal roots (NLSR)	
		χ^2	*p-value**	χ^2	*p-value**	χ^2	*p-value**
Cultivar (C)	2	7.33	0.0256*	16.60	0.0002*	28.36	<0.0001*
Ploidy (P)	1	25.46	<0.0001*	10.96	0.0009*	43.44	<0.0001*
Temperature (T)	3	51.76	<0.0001*	63.63	<0.0001*	78.55	<0.0001*
C × P	2	5.57	0.0618	2.44	0.2953	0.93	0.6287
C × T	6	11.71	0.0687	24.84	0.0004*	19.50	0.0034*
P × T	3	11.71	0.0085*	15.74	0.0013*	14.92	0.0019*
C × P × T	6	25.36	0.0003*	10.03	0.1235	6.44	0.3760
Factor				**RL** (cm)	**CL** (cm)		**NLSR**
C [a]		AG4051		4.59 a	2.00 a		3.24 a
		IAC Nelore		4.94 a	1.88 a		2.91 a
		CD384		2.96 b	1.05 b		1.99 b
P [b]		Haploid		2.50 b	1.18 b		1.81 b
		Diploid		5.00 a	1.88 a		3.18 a
T [a]		20		1.77 c	0.53 c		1.08 c
		25		5.34 b	1.73 b		2.89 b
		30		5.98 a	2.50 a		3.64 a
		35		3.50 b	1.88 a		3.38 a

* Significant effects at 5% probability level according to the chi-square test. [a, b] Mean values followed by the same letter in a column do not differ from each other at 5% significance level according to Wald's contrasts and the chi-square test, respectively.

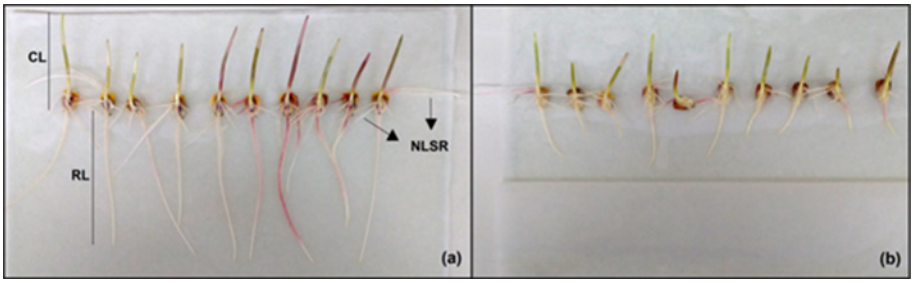

Figure 1. Traits of diploids (a) and possible haploids (b) classified by the *R1-Navajo* marker using the AG4051 cultivar crossed with the TAIL9 inducer and germinated at 30 °C. Features include radicle length (RL), coleoptile length (CL), and the number of lateral seminal roots (NLSR).

of 30 °C was selected as the optimal T to validate the use of the seedling traits as a procedure to eliminate remaining diploids selected by *R1-Navajo*. This T, as well as 25 °C, most clearly showed differences in the development of significant interactions in the adopted model; however, the germination rate obtained at 30 °C was 100%. Additionally, another factor that influenced the choice of T was the percentage of the seedlings that survived after transplantation. The seeds that germinated at 30 ºC had better development after transplantation, resulting in a higher survival percentage (88.33%) compared to T of 25 °C (80%), with losses of 7 and 12 plants, respectively.

Use of seedling traits in haploid identification among diploid seeds

To determine the use of seedling traits, described by Chaikam et al. (2017), a cut-off point (*threshold*) was established in the present study for each variable analyzed. The search for an ideal cut-off point enabled the reduction in false discoveries and increase in positive discoveries. In this study, we used individual cut-off points because *deviance* differences were found between the cultivars used in crosses. The obtained cut-off points, considering all crosses, displayed a greater change when RL was analyzed, with values between 2.1 and 5.6 cm (Table 2). For CL and NLSR, the variation was smaller, reaching values from 1.8 to 2.8 cm and 2 to 3 roots, respectively. However, it appeared that the higher the cut-off point for RL, the higher the cut-off point for CL and NLSR when cultivar AG4051 was used in the crosses.

The highest AUC values were obtained for the descendants of the crosses with cultivar AG4051when RL and CL were evaluated (Table 2 and Figure 2). Although lower values were determined for crosses with the cultivars CD384 and IAC Nelore, the corresponding CL and RL values indicate that for these two cases, the probability of correctly classifying haploids and/or diploids using the seedling traits was extremely small. The rates of false discoveries (FDR) and false negatives (FNR) were more satisfactory when RL was evaluated for the cross AG4051 × TAIL9 and when RL and NLSR were evaluated for the cross IAC Nelore × TAIL9. These results are in agreement with those obtained for accuracy, which corresponded to the proportion of both positive and negative hits based on the cut-off point because the highest accuracy levels were obtained for the crosses that had the lowest FDR and FNR values (Table 3 and Figure 3).

Evaluation of the agreement between the *gold standard* (phenotype) and the diagnostic test based on the seedling traits indicated that the descendants of AG4051 × TAIL9 cross exhibited agreement when RL and CL were analyzed;

Table 2. Cut-off point and area under the curve (AUC) defined by ROC for three traits

	Cultivar	Cut-off point	AUC
Radicle length (cm)	AG4051	5.6	0.987
	IAC Nelore	2.1	0.533
	CD384	4.9	0.630
Coleoptile length (cm)	AG4051	2.8	0.927
	IAC Nelore	2.0	0.779
	CD384	1.8	0.510
Number of lateral seminal roots	AG4051	3.0	0.722
	IAC Nelore	2.0	0.796
	CD384	2.0	0.816

that is, the classification made after the cut-off point was analogous to the phenotypic classification (Table 3 and Figure 3). For CL, there was also agreement for the descendants of the IAC Nelore × TAIL9 cross. For NLSR, the crosses IAC Nelore × TAIL9 and CD384 × TAIL9 showed a classification based on the cut-off point equivalent to that observed for the phenotype. According to Byrt et al. (1993), the agreement of these crosses varies from moderate to excellent, considering the Kappa coefficient obtained for each.

Seedling trait evaluation is efficient in reducing remaining diploids identified by the *R1-Navajo* marker, which is widely used in DH research because of the significant differences for all analyzed variables when comparing haploid and diploid seedlings. Chaikam et al. (2017) reported a small proportion of seedlings (approximately 10%) that could not easily be separated into haploids or diploids using seedling traits because they had intermediate traits; however, the authors still considered this methodology more precise than *R1-Navajo*.

McNemar's test was previously adopted by Melchinger et al. (2016) to compare test scores, such as *R1-Navajo*, *liguleless*, *glossy*, and resistance to *Ultrafox* herbicides using results obtained by flow cytometry as the *gold standard*. Silva et al. (2020) also used this test to compare the equivalence between stomatal guard cell length classifications and phenotype. In both studies, it was verified that, in some cases, the test did not indicate associations between the classifications, which is in agreement with our research.

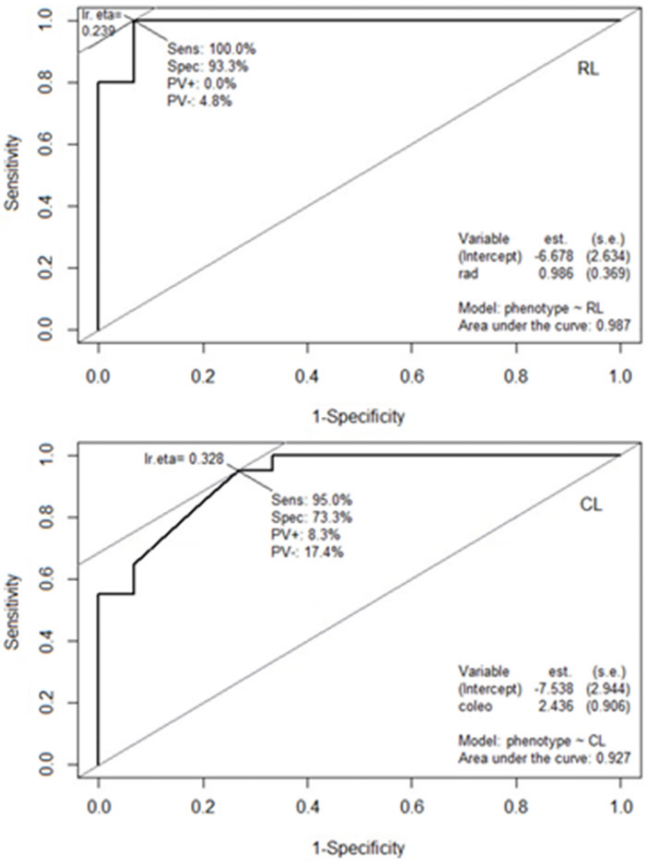

Figure 2. ROC curve for the descendants of the induction cross between the cultivar AG4051 and the inducer TAIL9 for radicle length (RL) and coleoptile length (CL). ROC curves were generated with the *ROCR* and *Epi* package in the *R* software. lr.eta: probability associated with the cut-off; Sens: sensitivity; Spec: specificity; PV+: positive predictive value; PV-: negative predictive value; est: parameter estimates.

This leads us to infer that the method based on seedling traits can be used to separate haploids from diploids, thus reducing the diploids that remained after selection by *R1-Navajo*. In general, as observed by Chaikam et al. (2017), seedlings with longer radicles tend to have larger

Table 3. False discovery rate (FDR), false negative rate (FNR), accuracy, Kappa coefficient, and McNemar test values obtained for haploid and diploid classification from of the cut-off points established by the ROC curve

Variable	Cross[a]	FDR (%)	FNR (%)	Accuracy (%)	Kappa Coefficient [b]	Kappa *p-value*	McNemar *p-value*
RL (cm) [c]	1	9.09	0	94.29	0.88 excellent	1.51×10^{-7} *	0.4795
	2	9.38	3.33	88.24	0.28 moderate	0.0836	0.6171
	3	87.50	94.74	21.88	-0.43 weak	0.0018 *	0.0455 *
CL (cm) [c]	1	20.83	5.00	82.86	0.63 very good	0.0001 *	0.2207
	2	6.90	10.00	85.29	0.36 moderate	0.0339 *	1.0
	3	16.67	73.68	53.13	0.16 weak	0.1850	0.0019 *
NLSR [c]	1	40.63	5.00	60.00	0.09 weak	0.3830	0.0033 *
	2	9.09	0	91.18	0.37 moderate	0.0054 *	0.2482
	3	25.00	5.26	78.13	0.52 good	0.0018 *	0.1306

[a] 1- AG4051 × TAIL9; 2- IAC Nelore × TAIL9; 3- CD384 × TAIL9
[b] Classification according to Byrt et al. (1993).
[c] See acronymous in Table 1.

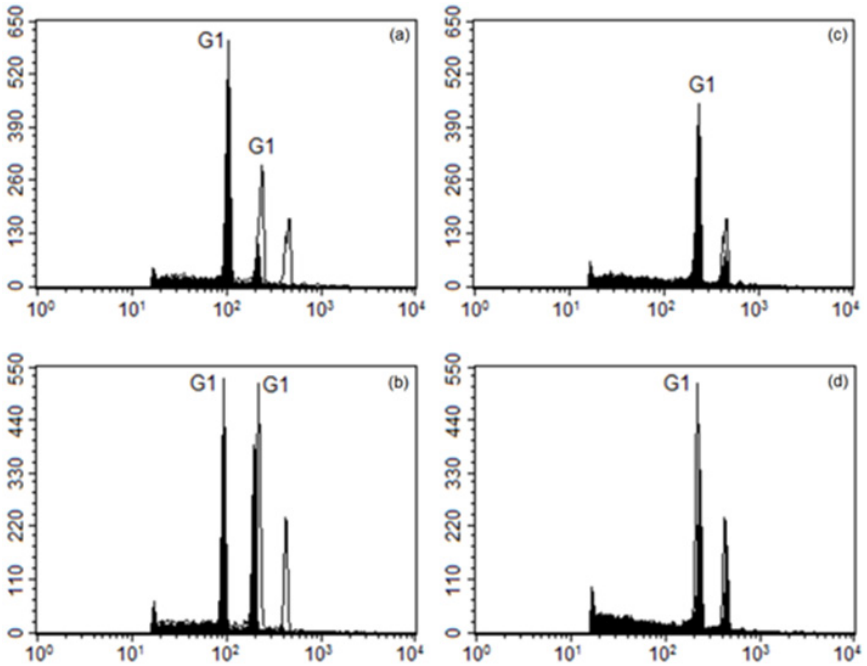

Figure 3. Histograms obtained from flow cytometry in the BD FACSCalibur flow cytometer with the Cell Quest software. On the left, haploid plants; on the right, diploid plants. The white histograms represent the standard and the black histograms the samples analyzed. The x-axis represents the intensity of relative fluorescence and the y-axis the number of nuclei read.

coleoptiles and are identified as diploids by the *gold standard*. Despite the need to obtain the cut-off point individually for each genotype, making the process more time-consuming, it is worth noting that the use of seedling traits to discard false positives, identified by *R1-Navajo*, can be promising in the DH line development, since discard can be performed before chromosomal duplication, thereby reducing costs, labor, time, and greenhouse space, besides being a simple and non-destructive methodology.

Coincidence between phenotype and flow cytometry

In the right-hand-side histograms (Figures 3c and 3d), the G1 peak of the sample coincided with that of the standard because both belong to the same species. Thus, plants that showed histograms of this type were classified as diploids by flow cytometry. Conversely, histograms of plants classified as haploids by flow cytometry had a G1 peak for the sample that is shifted to the left and a G2 peak superimposed on the G1 peak of the standard (Figures 3a and 3b).

After analyzing all histograms and obtaining the classification of the ploidy of the plants by flow cytometry, a 96.13% coincidence was obtained between the classification by flow cytometry and classification based on the plant phenotype. Despite the high coincidence between phenotype and cytometry, it is worth noting that phenotypic classification can generate uncertainties resulting from intermediate traits shown by the plants. Thus, a second methodology should be applied or intermediate plants should be discarded. Battistelli et al. (2013) stated that it is possible to eliminate diploids considering the vigor in adult plants.

Among the methods analyzed in the present study, flow cytometry was efficient in confirming the ploidy of the plants. This methodology is widely used in DH studies but requires expensive equipment and a trained workforce (Choe et al. 2012, Silva et al. 2020). The methodology using seedlings was also efficient in separating haploids and diploids, and its cost is low compared to that of flow cytometry because it does not need expensive equipment or technical expertise.

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