



Effects of donor and inducer genotype and endosperm texture on haploid induction in maize assessed using *R1-Navajo*

Mariana Martins Marcondes¹ · Matheus Lucas Schuck¹ · Robson Marcelo Rossi¹ · Vanderlei Aparecido de Lima¹ · Rodrigo Gomes¹ · Carlos Alberto Scapim¹ · Marcos Ventura Faria¹

Received: 26 June 2025 / Accepted: 16 April 2026 / Published online: 29 April 2026
© The Author(s) 2026

Abstract The double haploid (DH) technique in maize (*Zea mays* L.) relies on in vivo haploid induction, in which the identification of haploid seeds and seedlings represents a critical step in this technique that determines the efficiency of DH line production. This study aimed to evaluate the effects of endosperm texture, donor genotype, and inducer on putative haploid rates based on the *R1-navajo* (*R1-nj*) marker and the use of guard cell length and stomatal density as a tool to identify diploids among putative haploids. Eleven hybrids were assessed as donor genotypes and were pollinated with ten haploid inducers. The response was the number of putative haploid seeds

based on *R1-nj* phenotype, evaluated across the total number of seeds. Previously classified seeds of two induced maize hybrids were grown, and epidermal impressions of the abaxial face of maize leaves were used to measure guard cell length (GCL) and stomatal density (SD). Plants were classified according to the real ploidy by the gold standard evaluation. The three-way cross inducer (UH400×UH401)×TAIL 9 achieved the highest putative haploid rate (PHR) with five donor hybrids. The magnitude of the differences presented for GCL and SD were sufficient for the efficiency in the classification of ploidy in maize by means of logistic regression. The AUC values obtained were 0.916 for GCL and 0.941 for SD, indicating that both tests under study can be integrated into maize breeding programs, which can be used for proper disposal of diploids among putative haploids.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10681-026-03733-6>.

M. M. Marcondes · R. Gomes · M. V. Faria
Departamento de Agronomia, Universidade Estadual do Centro-Oeste/UNICENTRO, Guarapuava, Paraná, Brazil

M. L. Schuck (✉) · C. A. Scapim
Departamento de Agronomia, Universidade Estadual de Maringá/UEM, Maringá, Paraná, Brazil
e-mail: mlschuck@uem.br

R. M. Rossi
Departamento de Estatística, Universidade Estadual de Maringá/UEM, Maringá, Paraná, Brazil

V. A. de Lima
Departamento de Química, Universidade Tecnológica Federal do Paraná/UTFPR, Pato Branco, Paraná, Brazil

Keywords *Zea mays* L. · Navajo phenotype · Maternal haploids · Guard cell length · Stomatal density

Introduction

In maize (*Zea mays* L.) breeding programs focused on the production of double haploid (DH) lines, the in vivo method for obtaining maternal haploids involves four key steps: (1) haploid induction, achieved by the crossing of a maternal haploid inducer pollen donor with the donor genotype, which

produces maternal haploid seeds; (2) identification and separation of haploid seeds; (3) chromosome doubling of the haploid plants using antimitotic agents; and (4) self-pollination of the doubled plants to produce double haploid lines. Compared to the conventional method that involves successive generations of self-pollination, the DH technique enables the development of homozygous inbred lines in a single generation, reducing the time and resources required for maize breeding (Chaikam et al. 2019).

The haploid induction rate (HIR) is determined by the ratio of haploid seeds to the total number of seeds produced. Higher HIR values are essential for the efficiency of DH-based breeding programs and allow the production of doubled haploid lines on a large scale (Silva et al. 2023). The HIR characteristic is first influenced by the haploid inducer genotype. In the early use of DH in maize breeding, the Stock 6 line was widely used as inducer, with an average production rate of 2–3% haploid seeds. The later breeding for haploid induction resulted in HIR ranging from 8 to 15% in haploid inducer lines such as RWS, UH400, and TAIL (Coe 1959; Prasanna 2012).

In addition, the HIR is significantly influenced by the donor's genetic background (Silva et al. 2020). De La Fuente et al. (2018) observed putative haploid rates ranging from 2.6 to 30.5% across 30 hybrids pollinated with the same inducer RWS/RWK-76. Among other potential factors, anthocyanin inhibitor alleles (*C1-I*, *C2-Idf*, and *in1-D*, which act epistatically on *R1-nj*) present in the donor can prevent the formation of aleurone layer pigmentation. Generally, flint maize harbors anthocyanin inhibitor genes at a higher frequency than dent maize, making the identification of haploid seeds more difficult in flint or flint×dent hybrids (Eder and Chalik, 2002; Chen et al. 2024). Given the complex interaction that inducers and donor germplasm have on the haploid induction rate, studies are needed to determine the dependence between these factors, especially for tropical conditions (Ren et al. 2022; Trentin et al. 2023). Identifying significant interactions could enable the use of specific inducer lines for the donor genotypes, thus reducing the costs associated with haploid selection.

Furthermore, in the production of double haploids lines reliable methods are required to confirm the haploidy of plants from induction crossings, prior to duplication, in the seed or seedling stages (Dang et al. 2012; Melchinger et al. 2016). Additionally, methods

are needed that can eliminate diploid plants that perhaps were not eliminated by previous processes, before the lines self-fertilization phase. This assessment would minimize the number of false positives, time, investment and unnecessary labor. Reports using genes that express purple or red coloring in the stem and leaf sheath (Rober et al. 2005; Li et al. 2009) and oil content in seeds (Melchinger et al. 2014), are examples of methods tested to be incorporated into breeding programs that use the DH technique.

Among the approaches investigated for ploidy discrimination, stomatal morphological traits have been explored (Pires et al. 2023). The length of the stomata guard cells has been reported to correlate with DNA content in coffee (Mishra 1997), *Chrysanthemum* sp. (Wang et al. 2014), and *Clematis* sp. (Wu et al. 2013), suggesting its potential use to differentiate ploidy in plants. Studies have also evaluated guard cell length and stomatal density in maize leaves to discriminate diploid and double-haploid plants (Choe et al. 2012; Molenaar et al. 2019; Silva et al. 2020; Ribeiro et al. 2022). In this sense, techniques based on stomata guard cells for discrimination of ploidy in maize have been indicated as reliable, non-destructive and applicable to a wide range of germplasms. The demand for simple and effective procedures for identification of haploids is increasing. Ideally, these methods should be applicable during seed or seedling phases, across different germplasms and be fast with low operational cost.

Therefore, this study aimed to evaluate the effects of endosperm textures of donors, donor and inducer genotypes on haploid induction rates, based on the *R1-nj* phenotype in maize seeds and the use of guard cell length and stomatal density as a tool to identify diploids among putative haploids.

Material and methods

Plant material

Seeds were obtained from the pollination of eleven maize hybrids, used as donor genotypes (female parents), with ten haploid inducer maize genotypes (male parents). The donor genotypes comprised ten single hybrids and one three-way hybrid. The donor hybrids selected are commercially important and widely adopted in maize cultivation in Brazil. These

Table 1 Hybrids used as donor genotypes in the evaluation of putative haploid rate (PHR) in maize

Hybrid	Company	Type	Texture
30A68 HX	Morgan	SH	Semi-flint
AG 8780 PRO3	Bayer	SH	Semi-dent
AS 1555 RR	Bayer	SH	Semi-flint
AS 1677 PRO3	Bayer	SH	Semi-flint
BG 7060	Corteva	TH	Semi-flint
Fórmula	Syngenta	SH	Flint
NS 50 PRO	Syngenta	SH	Semi-flint
NS 92 PRO	Syngenta	SH	Semi-flint
P1630 HX	Corteva	SH	Semi-dent
P4285 YHR	Corteva	SH	Flint
SYN 7205	Syngenta	SH	Semi-flint

Table 2 Maternal haploid inducers used in the evaluation of putative haploid rate (PHR) in maize

Inducer	Type	Origin
TAIL 7	IL	Tropical
TAIL 8	IL	Tropical
TAIL 9	IL	Tropical
TAIL 7×TAIL 8	SH	Tropical
UH400×UH401	SH	Temperate
(UH400×UH401)×TAIL 7	TH	Temperate/Tropical
(UH400×UH401)×TAIL 9	TH	Temperate/Tropical
Nidera Orig TAIL 1	IL	Temperate/Tropical
Nidera Orig TAIL 2	IL	Temperate/Tropical
Nidera Orig TAIL 3	IL	Tropical

IL: inbred line; SH: single cross hybrid; TH: three-way cross hybrid.

hybrids represent elite tropical germplasm and exhibited semi-dent, semi-flint and flint endosperm textures (Table 1).

SH: single cross hybrid; TH: three-way cross hybrid.

The evaluated haploid inducers were chosen due to their stable *R1-nj* expression and adaptability under tropical conditions (Table 2), frequently used by the breeding companies. They included: a single cross hybrid derived from the temperate lines UH400 and UH401, developed by the University of Hohenheim, Germany, from the KEMS (Krasnador Embryo Marker Synthetic) inducer (Liu et al. 2016); tropical inbred lines adapted TAILs 7, 8, and 9 (Tropically Adapted Inducer Lines), developed by

CIMMYT (International Maize and Wheat Improvement Center), Mexico, in collaboration with the University of Hohenheim, through backcrossing of the UH400 and RWS (Russian Inducer KEMS+WS14) inducers with tropical maize lines (Prasanna 2012; Liu et al. 2016); inducer inbred lines developed by Nidera Sementes, originating from TAILs 1, 2, and 3; and simple and three-way cross hybrids obtained from the UH400, UH401, TAILs 7, 8, and 9 inducers.

Haploidy-inducing crosses and phenotypic classification

The pollination field was cultivated during the 2016/2017 growing season in Abelardo Luz, SC, Brazil, and was conducted at Nidera Sementes, a commercial maize seed production company. The donor and inducer genotypes were arranged in 6 m rows per combination (donors×inducers), with inter-row and intra-row spacings of 0.90 m and 0.20 m, respectively. Haploid inducers were planted in an adjacent field. To ensure synchronizing flowering periods, inducer genotypes were sown in five sequential planting dates, spaced one week apart; the first and last sowings occurred one week before and 21 days after the donor genotypes, respectively. The ears of the donor genotypes were protected prior to silk emergence. During the flowering period, pollen from each inducer was bulk-collected using paper bags, and pollinations were performed manually.

The pollinated ears were harvested separately and the seeds produced were classified based on the absence/presence of purple coloration on the scutellum and the aleurone of seeds, result from the expression of the dominant *R1-nj* marker gene present in the inducer germplasm, as described by Nanda and Chase (1966). Therefore, seeds with purple endosperm and white embryos (uncolored) were considered putative haploids (Fig. 1). The total number of seeds per ear was obtained by summing putative haploid seeds, cross-pollinated diploids (with purple endosperm and purple embryo) and normal seeds (without purple pigmentation).

Stomata experiment procedures

Seeds classified previously obtained from the crosses among two maize single hybrids and ten haploid inducers (Table 3) were used for the



Fig. 1 Illustrative schematic of *R1-navajo* phenotypes: **A** putative haploid seeds (purple endosperm and colorless embryo), **B** cross-pollinated diploid seeds (purple endosperm and purple embryo), and **C** normal seeds (no purple pigmentation)

evaluation of stomatal density (SD) and guard cell length (GCL). Random samples were taken from each ploidy fraction, and the number of seeds from each cross was used to implement the experiment. Seeds from each cross were individually placed to germinate on paper rolls, under controlled temperature of $26 \pm 1^\circ\text{C}$ for 96 h in a BOD chamber (Prasanna 2012). Seedlings were transplanted in 50-cell plastic trays (diameter 4.8×9.0 cm height) containing vegetable soil substrate. The trays were kept in a greenhouse for 10 days to acclimatize the plants. After 10 days, the seedlings were transplanted to the field, in lines of 5 m in length, spaced 0.8 m apart and spacing of 0.2 m between

plants. The field experiment was carried out in the 2016/2017 crop season in Guarapuava, Paraná, Brazil.

Epidermal impressions of guard cell and stomata were obtained according to Kim et al. (2010) and replicated by Choe et al. (2012) using cyanoacrylate glue. The slides containing the epidermal impressions of the abaxial face were made from leaf samples (0.5×1 cm), collected in duplicate in the leaf's median region, one on each side of the rib, from the fully expanded leaves of the 3rd and 4th nodes counted from the base. The images were captured in a light microscope model Primo Star®, with a camera attached, by the software Zen Lite 2001. For the evaluation of the GCL, the images were captured with a $40\times$ objective, and the measures were obtained from four stomata chosen at random, with the aid of the ImagePro®Plus software. For the evaluation of SD, the images were captured with a $10\times$ objective, and the number of stomata was counted from three different sample points (0.3×0.3 mm²), chosen at random, also with the aid of the ImagePro®Plus software.

To evaluate the true ploidy, the plants were evaluated at the anthesis stage using the gold standard classification, based on the plants visual assessments according to the tassel vigor and fertility (Melchinger et al. 2013). Less vigorous plants, of short stature, with thinner and erect leaves, associated with reduced tassel fertility were classified as haploid, and vigorous and fertile plants as diploid (Fig. 2). All plants, from each crossing and from each ploidy fraction, were tagged and numbered so that the evaluations could be compared.

Table 3 Number of seeds (N) putative haploids (PH) and putative diploids (PD), from each cross, used to implement the experiment

Inducer	Hybrid	N		Hybrid	N	
		PH	PD		PH	PD
TAIL 7	AS 1555 RR	50	50	P1630 HX	50	50
TAIL 8	AS 1555 RR	50	50	P1630 HX	50	50
TAIL 9	AS 1555 RR	20	50	P1630 HX	50	50
TAIL 7 $\times$ TAIL 8	AS 1555 RR	50	50	P1630 HX	50	50
UH400 $\times$ UH401	AS 1555 RR	50	50	P1630 HX	50	50
(UH400 $\times$ UH401) $\times$ TAIL 7	AS 1555 RR	50	50	P1630 HX	50	50
(UH400 $\times$ UH401) $\times$ TAIL 9	AS 1555 RR	50	50	P1630 HX	50	50
Nidera Orig TAIL 1	AS 1555 RR	20	50	P1630 HX	50	50
Nidera Orig TAIL 2	AS 1555 RR	20	50	P1630 HX	50	50
Nidera Orig TAIL 3	AS 1555 RR	50	50	P1630 HX	50	50



Fig. 2 Gold standard evaluation of plant phenotype. **A** Phenotypic difference in vigor between diploid and haploid plants; difference in tassel fertility between **B** diploid and **C** haploid plants

Statistical analysis of the *RI-nj* data

The response variable was the number of putative haploids (y_{ijkl}) out of the total number of seeds (n_{ijkl}) obtained from individual ears for each donor $\times$ inducer combination. Thus, y_{ijkl} follows a binomial distribution with the probability of success π_{ijkl} (Putative Haploid Rate-PHR)

$[y_{ijkl} \sim \text{Binomial}(\pi_{ijkl}, n_{ijkl})]$, using a logit link function, as in equation:

$$\text{logit}(\pi_{ijkl}) = \eta_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_{k(j)} + \tau_{i(j)} + \omega_{i(jk)}$$

where η_{ijkl} is the linear predictor for l -th ear from k -th donor genotype within j -th endosperm texture and i -th inducer genotype, μ is the constant inherent in

the data, α_i represents the effect of the i -th inducer, β_j denotes the effect of the j -th endosperm texture, $\gamma_{k(j)}$ is the effect of the k -th donor genotype within the j -th endosperm texture, $\tau_{i(j)}$ is the effect of the i -th inducer genotype within the j -th endosperm texture, and $\omega_{i(jk)}$ is the effect of the i -th inducer genotype within the j -th endosperm texture and k -th donor genotype. The model was fitted using the 'glm' function from the 'stats' package (R Core Team 2025).

The model that provided a better fit to the data was subjected to residual analysis using diagnostic plots with simulated envelopes, employing the 'hnp' package (Moral et al. 2017). Overdispersion was detected, as the amount of variation in the data exceeded that expected by the model. Consequently, the estimated standard errors and confidence intervals were corrected using the quasibinomial model, where the variance is given by $\phi\pi_{ijkl}(1 - \pi_{ijkl})$, with ϕ as the dispersion parameter (Demétrio et al. 2014).

Sequential deviance analysis was performed, where factors of the model were assessed using the F statistic. The pairwise contrasts of the estimated means within each level of the factors were estimated, and inference was made using the Wald test. The p-values were adjusted using the Bonferroni correction to maintain the global error rate at $\alpha = 0.05$. The means on the linear predictor scale were then converted to probabilities using the inverse of the link function, as in equation:

$$g^{-1}(\pi_{ijkl}) = \frac{e^{\eta_{ijkl}}}{1 + e^{\eta_{ijkl}}}$$

Asymptotic confidence intervals for the estimated contrasts were obtained using the 'emmeans' package in the R environment (R Core Team 2025).

Accuracy of ploidy identification using *RI-nj*

From the ploidy established by gold standard, diploids confirmed as true were categorized as true positives (TP) and haploids that were confirmed as true were categorized as true negatives (TN). The true haploids classified in the diploid fraction were called false positives (FP) and the true diploids in the haploid fraction were called false negatives (FN). The numbers of TP, TN, FP and FN were adjusted in each population based on total number of haploids and diploids identified by the gold standard. These data were also used to calculate the false discovery rate

(FDR) and the false negative rate (FNR) (Melchinger et al. 2013).

Statistical analysis of the guard cell length and stomatal density

The stomata data were analyzed by Generalized Linear Models (GLM) with gamma distribution and log link function, were used to test the significance of the differences in GCL measurements between haploid and diploid plants, as well as between hybrids and haploid inducers. For the variable SD, GLM was used with negative binomial distribution and log link function. The statistical model adopted for both variables was a saturated model, with fixed effect factors:

$$\log_e(\mu_{ijk}) = \eta_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij}$$

where $\log_e(\mu_{ijk})$ is the natural logarithm of the k -th observation with the i -th ploidy level of the crossing of the j -th, μ is constant, α_i is the effect of the i -th ploidy level, β_j is the effect of the j -th hybrid and $\alpha\beta_{ij}$ is the effect of the interaction of the i -th ploidy level and the j -th hybrid. The factors were tested using the likelihood ratio chi-square test and comparisons between means were made using the Wald test and Bonferroni correction at the 5% significance level. Statistical analyzes were performed using the R software (R Core Team 2025).

In order to verify the use of the variables GCL and SD in the identification of diploids plants, logistic regression was used. Cut off points for ploidy classification were established using the Receiver Operating Characteristic Curve (ROC curve). The probability value associated with the cutoff, sensitivity (SENS), specificity (SPE), positive predictive value (PPV), negative predictive value (NPV), and the area under the ROC curve (AUC) were calculated. These metrics were used to quantify the accuracy of GCL and SD in differentiating haploid and diploid plants based on gold-standard ploidy classification.

Results and discussion

A total of 240,285 seeds were evaluated across 1,394 ears. The number of ears per donor×inducer combination ranged from 0 to 30 (Supplementary Fig. 1). Because sample size directly affects the confidence

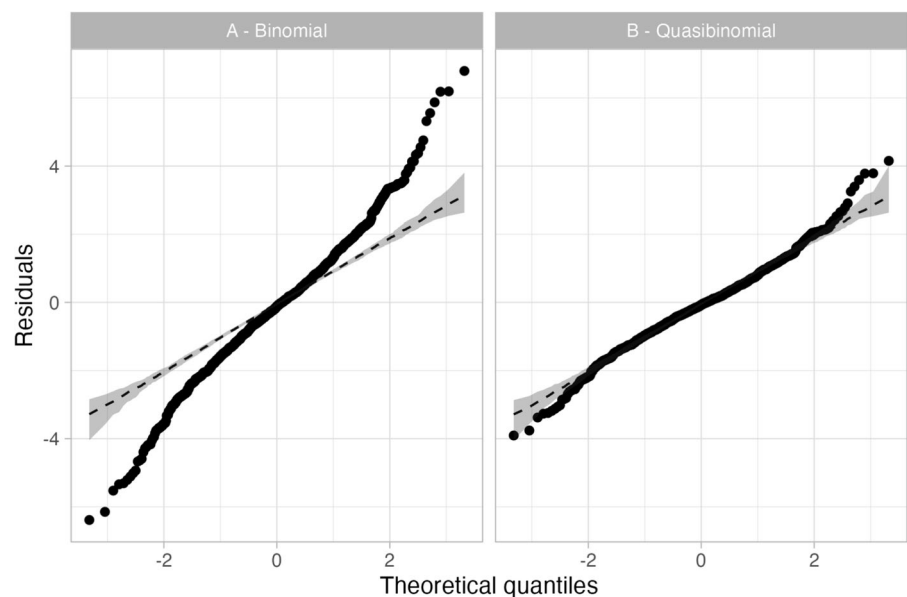
interval, it varied according to the number of ears obtained per donor $\times$ inducer combination. Larger sample sizes provided narrower confidence intervals for the PHR, increasing the statistical power to detect significant differences. Eight crosses did not produce any seeds, most likely due to a lack of flowering synchrony between the inducers and donors, so their means and contrasts involving these means could not be estimated. These crosses were: BG 7060 $\times$ TAIL 7, P4285 YHR $\times$ [TAIL 9, UH400 $\times$ UH401, (UH400 $\times$ UH401) $\times$ TAIL 9 and Nidera Orig TAIL 1], and SYN 7205 $\times$ [TAIL 7, TAIL 7 $\times$ TAIL 8 and Nidera Orig TAIL 1]. A total of 15,254 putative haploid seeds were obtained, with an observed average PHR of 6.35% in the experiment, ranging from 0.05% to 18.63% across the donors $\times$ inducers combinations.

The half-normal plots provide strong evidence in favor of the model that accounts for overdispersion, with most residuals falling within the simulated envelope (Fig. 3). Thus, for this study the quasibinomial model provided a better fit and therefore is more appropriate for estimating standard errors of the estimated means. The estimated dispersion parameter for the quasibinomial overdispersion model was 2.67, which indicates that the data exhibit greater variability than expected under the binomial assumption. This overdispersion may result from heterogeneity among observations that was not explained by the model covariates

(Demétrio et al. 2014). When overdispersion is ignored, the standard errors of the estimated means are underestimated, inflating the type I error rate.

The sequential deviance analysis for PHR revealed significant effects for all evaluated factors, as well as for their interactions terms ($p < 0.05$) (Supplementary Table 1). Trentin et al. (2022), using seven groups of inducer genotypes crossed with five groups of donor genotypes from different backgrounds, observed significant interactions between inducers and donors in the haploid induction rate. On the other hand, Prigge et al. (2011) also found genotypic effects between inducers and donor germplasms, although no significant interactions were reported. Despite the genotypic effects, the environment can affect induction efficiency (Silva et al. 2023). Factors such as temperature, radiation, and humidity can influence both the expression of the *R1-nj* marker and the haploid embryo formation. Thus, the single season experiment limits the ability to capture environmental variability affecting haploid induction. Future studies should evaluate the donor $\times$ inducer combinations across multiple seasons or locations to better understand environmental effects and genotype $\times$ environment interactions influencing PHR. A broader sampling of donor genotypes within texture classes also would help confirm these patterns across diverse germplasm in future studies.

Fig. 3 Diagnostic plots with simulated envelopes of deviance residuals for **A** binomial and **B** quasibinomial logit models for the putative haploid rate (PHR) in maize



Performance of haploid inducers

The interaction plot for donor genotypes and inducers is also presented in Fig. 4. The inducer (UH400×UH401)×TAIL 9 exhibited the highest estimated mean when used as a haploid inducer in combination with five donor hybrids, being statistically superior to the lower-performing inducers (Supplementary Table 2). The inducer (UH400×UH401)×TAIL 9 was superior when combined with five donor hybrids. As a three-way cross hybrid, the accumulation of alleles at distinct loci responsible for haploid induction may explain the PHR observed for this inducer (Chaikam et al. 2019). Although PHR represents an indirect estimate, putative haploids classified using *R1-nj* are related to the true haploid induction rate and can serve as a basis for evaluating the efficiency of different inducers (Couto et al. 2015).

For the donor hybrid NS 50 PRO, the single hybrid inducer TAIL 7×TAIL 8 demonstrated the

highest haploid induction capacity (7.98%), outperforming the three inducers with lower haploid induction ability, including the three-way cross hybrid (UH400×UH401)×TAIL 9 (2.80%), which was superior in other crosses. For the hybrids AS 1677 PRO3 and SYN 7205, the inducer UH400×UH401 exhibited the highest PHR. The hybrids NS 92 PRO and P4285 YHR did not demonstrate a significant difference between the inducers.

The ability to induce maternal haploids is a quantitative trait controlled by multiple loci (Trentin et al. 2023). Two key genes have been successfully characterized: (1) a gene identified by three independent research groups, known as *MATRILINEAL* (*MTL*), *NOT LIKE DAD* (*NLD*), or *ZmPHOSPHOLIPASE-A1* (*ZmPLA1*) (Gilles et al. 2017; Liu et al. 2017; Kelliher et al. 2017), located within the QTL *qhir1* on chromosome 1; and (2) *ZmDMP* (Zhong et al. 2019), located within the QTL *qhir8* on chromosome 9. The QTLs *qhir1* and *qhir8* explain 66% and 20% of the genotypic variance on the haploid induction rate,

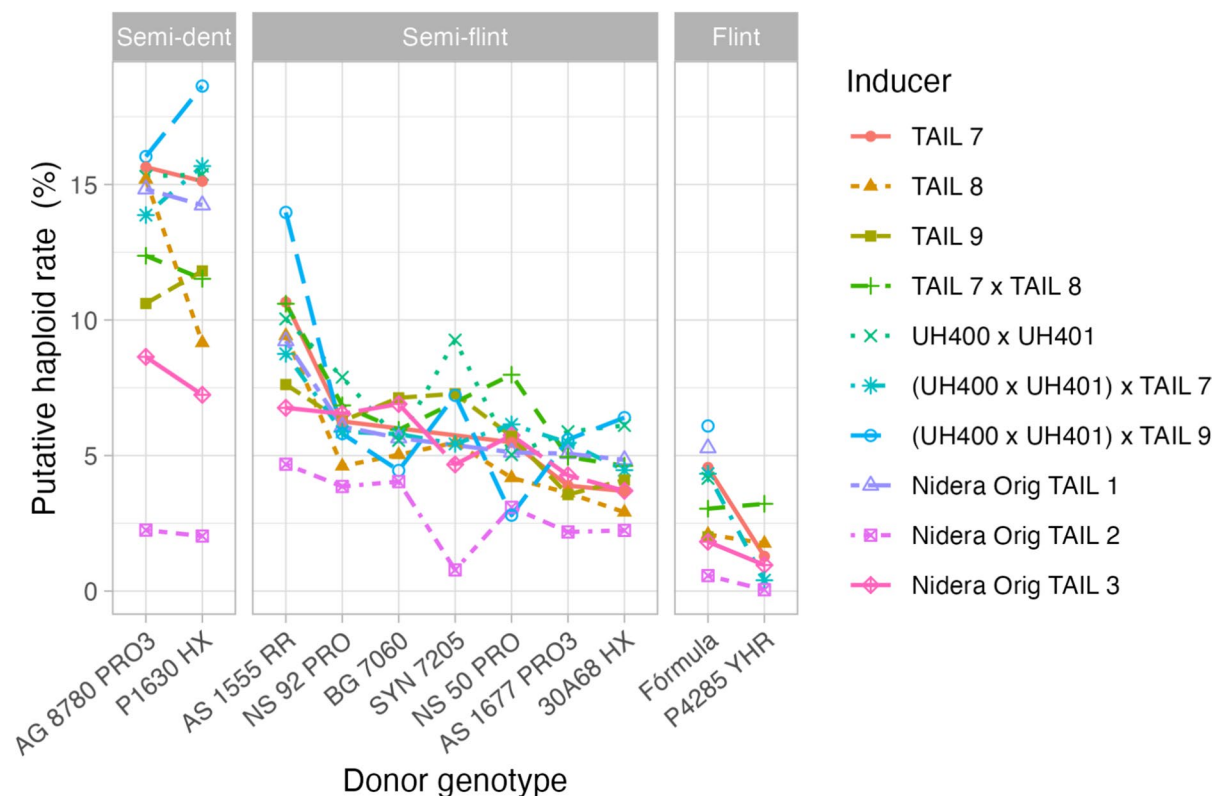


Fig. 4 Complex interaction between inducer genotypes and donor genotypes within endosperm textures in the putative haploid rate in maize

respectively (Prigge et al. 2012). Khammona et al. (2024) confirm the critical role of these QTLs for haploid induction rates through marker-assisted selection in segregating populations. While *qhir8* alone contributes a low haploid induction rate (0.15%), it enhances the rate by two to six times when combined with the mutant allele at the *MTL/NLD/ZmPLA1* locus, highlighting a synergistic effect between these genes (Zhong et al. 2019). Furthermore, Trentin et al. (2023) identified a major-effect QTL on chromosome 10 and several smaller-effect QTLs across six other chromosomes, reflecting the complex genetic architecture of this trait.

The biological mechanism underlying in vivo maternal haploid induction in maize is not yet fully understood. Two main phenomena have been proposed to explain the origin of haploid embryos: (i) single fertilization, in which only the polar nuclei are fertilized by the sperm nucleus and the inducer gamete fails to fuse with the egg cell, producing endosperm with normal paternal contribution and a haploid embryo; (ii) selective chromosome

elimination after double fertilization, in which chromosomes of inducer are eliminated during subsequent zygotic divisions (Gilles et al. 2017; Kelliher et al. 2017). These processes are functionally associated with inducer loci such as *MTL/NLD/ZmPLA1* and *ZmDMP*, which alter gamete behavior and can promote paternal genome elimination. The simultaneous occurrence of single fertilization and chromosome elimination may act in a complementary manner, highlighting that multiple biological pathways can contribute to haploid embryo formation (Zhong et al. 2019; Qu et al. 2024).

Verification of accuracy of ploidy identification using *RI-nj*

The gold-standard phenotypic evaluation of plants derived from putative haploid and diploid embryos demonstrated low FDR across the two hybrids evaluated (Table 4). For AS 1555 RR, FDR ranged from 0% to 4.44% (mean 1.81%), while false negative rate (FNR) varied from 0% to 15.69% (mean 7.28%). For

Table 4 Gold-standard confirmation of *RI-nj* based haploid/diploid embryo identification through phenotypic evaluation

Hybrid	Inducer	N	TP	TN	FP	FN	FDR (%)	FNR (%)
AS 1555 RR	TAIL 7	70	43	25	2	0	4.44	0.00
	TAIL 8	71	40	24	1	6	2.44	13.04
	TAIL 9	56	44	11	1	0	2.22	0.00
	UH400 x UH401	68	41	20	1	6	2.38	12.77
	TAIL 7 x TAIL 8	75	47	23	0	5	0.00	9.62
	(UH400 x UH401) x TAIL 7	61	43	10	0	8	0.00	15.69
	(UH400 x UH401) x TAIL 9	71	45	19	1	6	2.17	11.76
	Nidera Orig TAIL 1	59	46	12	0	1	0.00	2.13
	Nidera Orig TAIL 2	43	37	5	1	0	2.63	0.00
	Nidera Orig TAIL 3	77	47	27	1	2	2.08	4.08
P1630 HX		651	433	176	8	34	1.81	7.28
	TAIL 7	82	48	32	0	2	0.00	4.00
	TAIL 8	63	35	25	1	2	2.78	5.41
	TAIL 9	75	47	26	0	2	0.00	4.08
	UH400 x UH401	58	36	20	1	1	2.70	2.70
	TAIL 7 x TAIL 8	61	40	19	0	2	0.00	4.76
	(UH400 x UH401) x TAIL 7	63	45	16	1	1	2.17	2.17
	(UH400 x UH401) x TAIL 9	78	41	35	1	1	2.38	2.38
	Nidera Orig TAIL 1	67	41	22	0	4	0.00	8.89
	Nidera Orig TAIL 2	66	36	26	1	3	2.70	7.69
Total	Nidera Orig TAIL 3	65	36	25	1	3	2.70	7.69
		678	405	246	6	21	1.46	4.93
Total		1329	838	422	14	55	1.64	6.16

N: number of established plants evaluated for ploidy; TP: True Positives (diploids correctly identified); TN: True Negatives (haploids correctly identified); FP: False Positives (haploids misclassified as diploids); FN: False Negatives (diploids misclassified as haploids); FDR: False Discovery Rate; FNR: False Negative Rate.

P1630 HX, FDR ranged from 0% to 2.78% (mean 1.46%), and FNR ranged from 2.17% to 8.89% (mean 4.93%). Considering all combinations, the overall FDR was 1.64% and the overall FNR was 6.16%, indicating high accuracy of *R1-nj* haploid identification (Chaikam et al. 2016).

Effect of ploidy level and hybrid on guard cell length and stomatal density

From the visualization under a light microscope, visual differences could be noticed between haploid and diploid plants with respect to the stomata cell guard length (Fig. 5). Haploid plants showed shorter guard cells when compared to their diploid counterparts. Likewise, visual differences were detected in the stomatal density between ploidy plants. The leaves of haploid plants tended to have a higher number of stomata per area, compared to the leaves of diploid homologous plants (Fig. 6).

Statistical analyzes also revealed significant differences ($p < 0.05$) in GCL and SD between the phenotypes when this factor interacted with hybrids (Supplementary Table 3). Haploid plants had a mean GCL of 41.17 μm , while diploids had a mean of 58.16 μm (Table 5). In plants grouped as haploids and diploids, the GCL was greater in the hybrid P1630 HX (43.15 and 59.62 μm respectively). Across the source genotypes, the length of the haploid plants guard cells was always shorter when compared to the GCL of diploid plants. Thus, diploid plants had guard cell length mean 41% greater than those present in leaves of haploid plants.

For the two hybrids evaluated, SD in haploid plants was 25.61 and 24.58 stomata/ mm^2 , whereas diploid plants showed 13.62 and 14.04 stomata/ mm^2 for P1630 HX and AS 1555 RR, respectively (Table 6). Overall, haploid plants tended to have a higher number of stomata per area compared to diploids, in this case 25.09 stomata/ mm^2 compared

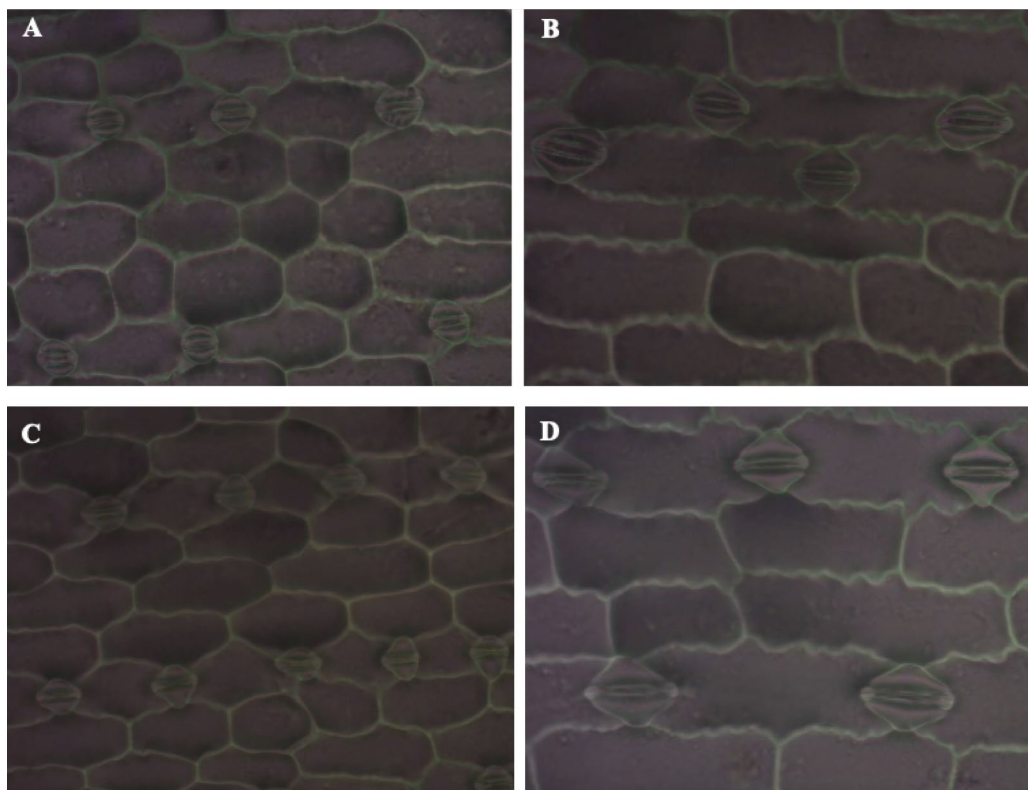


Fig. 5 Epidermal impressions of the abaxial face of maize leaves showing the size of stomata of haploid (A and C) and diploid (B and D) plants, from the crossings of haploid inducer

with the hybrids AS 1555 RR (A and B) and P1630 HX (C and D). Images captured in the 40 $\times$ objective

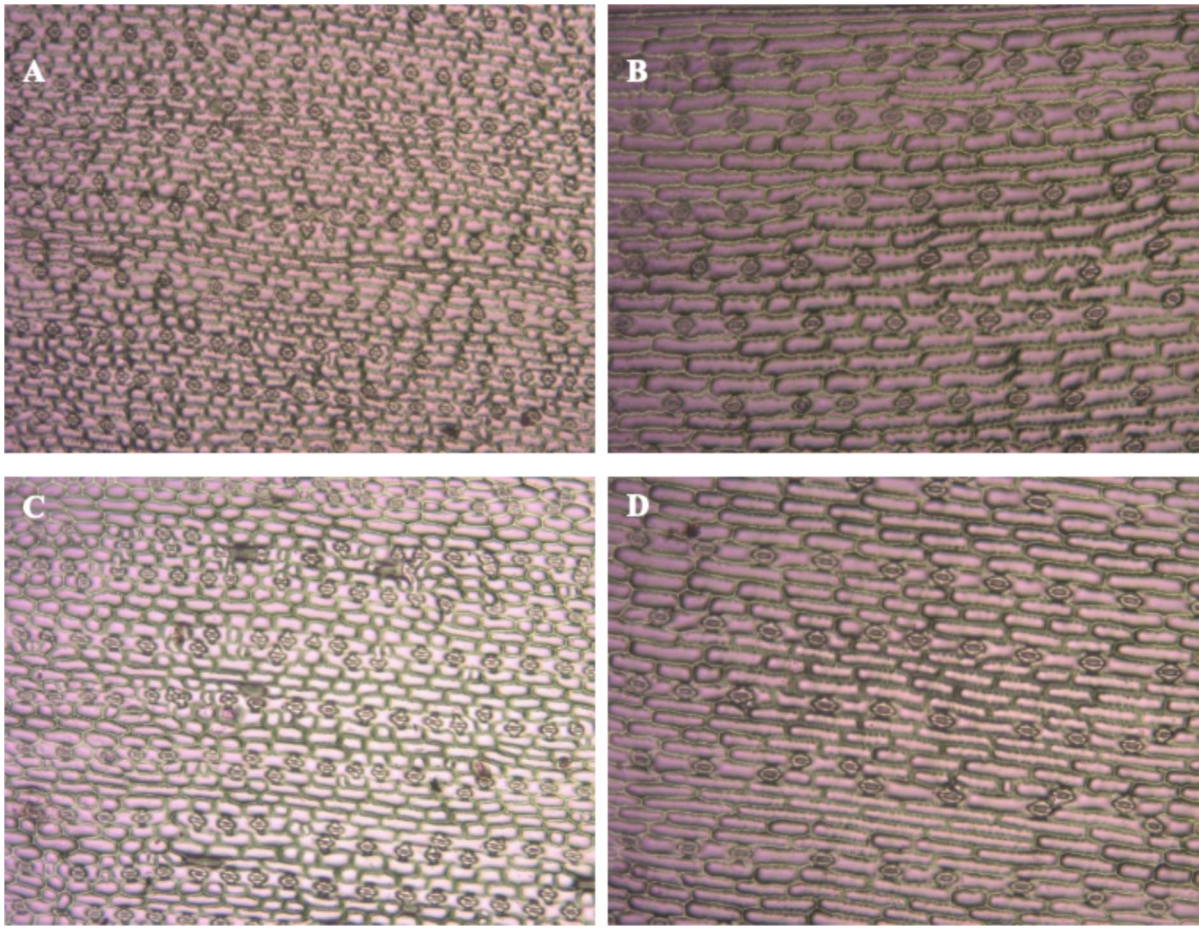


Fig. 6 Epidermal impressions of the abaxial face of maize leaves showing the stomatal density of haploid (**A** and **C**) and diploid (**B** and **D**) plants, from the crossings of the haploid

inducer with the hybrids AS 1555 RR (**A** and **B**) and P1630 HX (**C** and **D**). Images captured in the 10× objective

Table 5 Comparison between haploid and diploid phenotypes in interaction with hybrids for guard cell length (GCL) in maize leaves

Hybrid	Phenotypes		Delta ¹
	Haploid (μm)	Diploid (μm)	
P1630HX	43.15 ± 0.113 ^{aB}	59.62 ± 0.121 ^{aA}	16.47
AS1555RR	39.28 ± 0.121 ^{bB}	56.73 ± 0.111 ^{bA}	17.45
Mean	41.17	58.16	16.99

Means followed by the same lowercase letter in the column or uppercase in the row do not differ ($p < 0.05$) by using the Wald test and Bonferroni correction. ¹Delta: difference of means between phenotypes.

Table 6 Comparison between haploid and diploid phenotypes in interaction with hybrids for stomatal density (SD) in maize leaves

Hybrid	Phenotypes		Delta ¹
	Haploid (N/mm ²)	Diploid (N/mm ²)	
P1630HX	25.61 ± 0.111 ^{aA}	13.62 ± 0.058 ^{bB}	11.99
AS1555RR	24.58 ± 0.127 ^{bA}	14.04 ± 0.056 ^{aB}	10.54
Mean	25.09	13.83	11.26

Means followed by the same lowercase letter in the column or uppercase in the row do not differ ($p < 0.05$) by using the Wald test and Bonferroni correction. ¹Delta: difference of means between phenotypes.

with 13.83 stomata/mm², respectively. The deltas for GCL and SD can support ploidy classification, with mean difference of 16.99 μm and 11.26 stomata/mm² between haploids and diploids.

The negative correlation between stomata length and density is well known and has been demonstrated in stomatal conductance research (Franks and Beerling 2009). The control of stomata formation has been associated with both genetic and environmental factors. Thus, the length of guard cells has shown a relationship not only with the genome size (Beaulieu et al. 2008; Lomax et al. 2009), but also with hydric conditions to which the plants are submitted (Xu and Zhou 2008; Abdel and Stutzel 2016).

In a study on maize (Zhao et al. 2015), researchers observed that new stomata were developed continuously during the growth and leaf expansion of seedlings under normal growing conditions. This increase in the number of stomata kept the stomatal density relatively stable, while the stomata size (width and length) remained unchanged. These researchers also reported that maize seedlings that were subjected to severe drought conditions, significantly stimulated stomatal generation, which included a significant increase in stomatal density and a decrease in their length. This event was related to the premise that small stomata could maintain the opening of pores with lesser turgor pressure in the guard cells, compared to larger stomata. Thus, a higher density with reduced stomata size would inhibit water loss, guaranteeing water balance (Bosabalidis and Kofidis 2002).

Use of guard cell length for detecting diploids among putative haploids

The use of the expression of *RI-nj* marker is widespread in breeding programs that use double-haploid (Dang et al. 2012). However, the method can lead to separation of high numbers of diploids identified as haploids, the so-called false positives (Prigge et al. 2011; Melchinger et al. 2014; Chaikam et al. 2016). A method that can assist in the evaluation of plants, before duplication, reducing the number of false positives is the measurement of stomata length, since studies have shown that plants with different ploidy differ in relation to size of these cells in several species (Choe et al. 2012; Wu et al. 2013; Wang et al. 2014; Silva et al. 2019).

With adjusted logistic regression and probabilities (Supplementary Fig. 2), a cutoff point for the data set could be established using the ROC curve (Receiver Operating Characteristic Curve). ROC curves have been used in binary statistics and can introduce prior information obtained within the estimation process (gold standard), which reduces the uncertainties about inferences (Gonçalves et al. 2014). This point related to the tested measure is especially important when the diagnostic test is evaluated against a true underlying condition, in this case the real plant ploidy classified as haploid or diploid.

The ROC curve (Fig. 7) was generated for the complete set of GCLs relating them to the gold standard, real plant phenotype, regardless of the crosses from which the measurements were obtained.

The intersection point of the dotted lines marked a probability value that made it possible to obtain the estimated cutoff point. In this case, the probability adopted was 0.616 and estimated cutoff point for GCL of 48.71 μm was obtained (Table 7). From the cutoff point, the limit was established in which equal or greater measures of GCL classified the plant as diploid, and in contrast, lower measures classified the

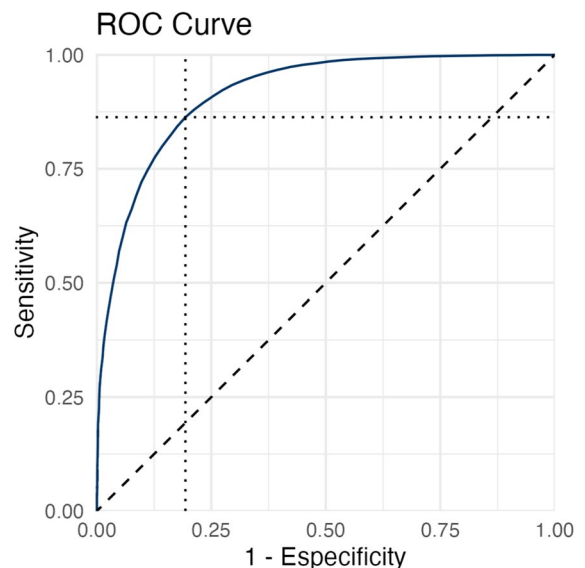


Fig. 7 ROC curve generated from the complete data set of guard cell length (GCL) of stomata in maize leaves. The intersection point of the dotted lines marks the probability associated with the ideal cutoff point and the continuous diagonal line represents the relationship of existing rates obtained by the model without the addition of variable evaluated response

Table 7 Probability values associated with the cutoff point, SENS, SPE, PPV, NPV and AUC obtained for the classification of putative diploids and putative haploids, from the cut-

off point defined for the guard cell length (GCL) of stomata in maize leaves, using the ROC curve

Variable	Probability	Cutoff Point (μm)	SENS (%)	SPE (%)	PPV (%)	NPV (%)	AUC
GCL	0.616	48.71	86.3	80.1	90.1	74.2	0.916

SENS: sensitivity; SPE: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve.

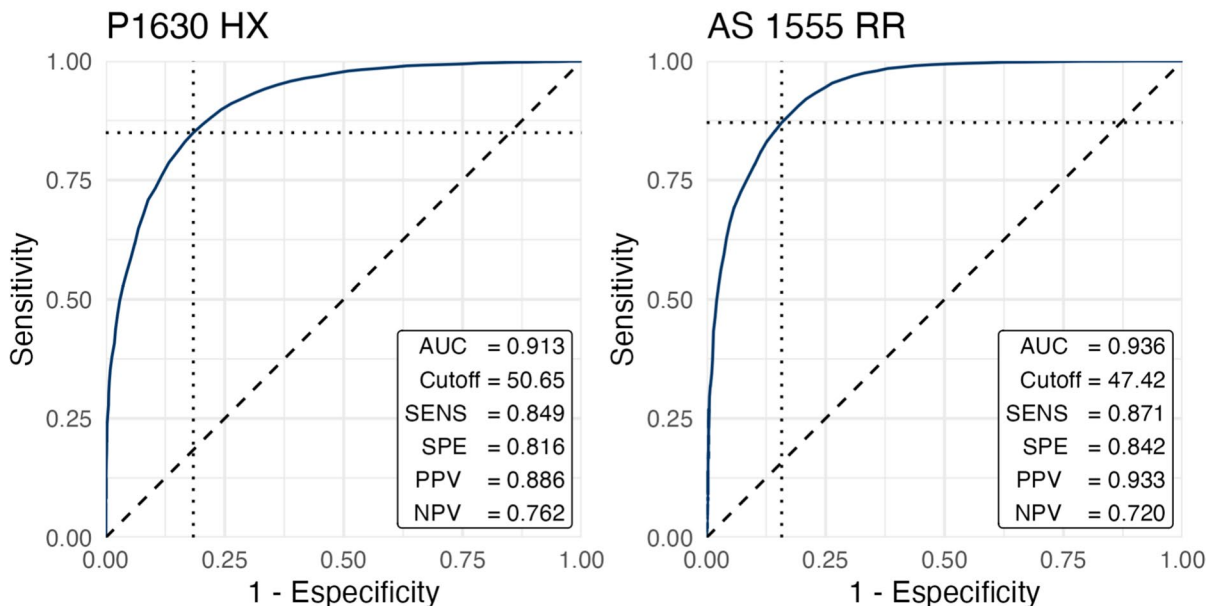
plant as haploid, for the data set in question. The contingency table generated from the complete data set for GCL is presented in Supplementary Fig. 3.

The values found for sensitivity of 86.3% and specificity of 80.1% at the established cutoff point were considered satisfactory, since the logistic model built classified them with high proportions of true positives and negatives. High proportions of PPV (90.1%) and NPV (74.2%) were also obtained. Indexes such as PPV (positive predictive value) and NPV (negative predictive value) indicate, in that order, the probability that a plant will actually be diploid when the test result is diploid, and the likelihood that a plant will actually be haploid when the test result is also haploid. Determining the area under the ROC curve value (AUC) is a simple and

quick method that indicates the performance of the diagnostic test in discriminating the studied classes (Gonçalves et al. 2014). Considering the AUC obtained of 0.916, it could be inferred that the GCL was a reliable diagnostic test to select diploids and haploids in this study.

Taking into account the interaction between hybrids and phenotypes, and the differences in GCL means observed across the crosses, the efficiency of ploidy classification could be affected if a single cutoff value were adopted. Therefore, a more specific approach was adopted by generating ROC curves and defining individual cutoff points for each cross (Fig. 8). Although specific cutoff values were estimated, the differences in predictive performance among the hybrids were relatively small, indicating

Guard Cell Length (GCL) - ROC Curves

**Fig. 8** ROC curves and associated classification metrics for guard cell length (GCL) in maize leaves, analyzed separately for the two hybrids

that the overall discriminative ability of the model was consistent across crosses.

Several researchers have used the GCL measure in research on plant breeding. Choe et al. (2012) proposed the evaluation of stomata guard cells for ploidy discrimination after chromosomal duplication, in double-haploid maize programs. Results showed that in the initial stages (leaf 2), it was possible to discriminate haploid plants from diploids and samples taken from leaf 5 were able to distinguish haploid, diploid and double haploid plants. The authors also comment that the technique may be more efficient than flow cytometry, as DH plants have chimeric tissues that can show both haploid and diploid peaks in the graph, requiring a high level of experience for the proper interpretation of results. In addition, stomata length measurement proved to be a faster and less expensive technique compared to flow cytometry.

The guard cell length proved to be efficient in detecting differences between haploids and diploids, and it can be a promising technique for use in genetic improvement, together with the phenotypic marker *R1-nj*, which allow the previous disposal of most diploid seeds (Molenaar et al. 2019; Silva et al. 2020). The greater disposal of diploid seeds by *R1-nj*, with the subsequent disposal of false positives in the initial stages by the GCL, maximizes the efficiency of this disposal, reducing labor, space and resources in breeding programs. GCL can also be used in breeding programs where the expression of the marker by anthocyanin is hindered or suppressed by the source genotypes (Chaikam et al. 2014).

Use of stomatal density to detect diploids among putative haploids

In the relation between stomata SD and plant ploidy, contrary to what was seen for the GCL, the probability of finding a diploid decreases as the number of stomata per area on the leaf increases (Supplementary Fig. 4). The cutoff point of 18.33 stomata/mm² from the ROC curve is explained (Fig. 9).

High values of sensitivity (89.4%) and specificity (87.2%) were found from the cutoff point defined for the total set of data obtained for SD (Table 8). The same occurred for the predictive values indicating the probability of detecting true (positive and negative), which were 93.5% and 80.1%, respectively. The Contingency Table for the ROC curve

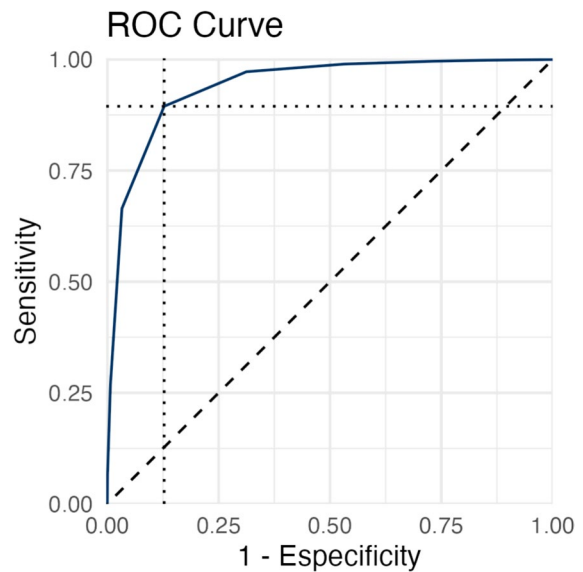


Fig. 9 ROC curve generated from the complete data set for the stomatal density (SD) in maize leaves. The intersection point of the dotted lines marks the probability associated with the ideal cutoff point and the dashed diagonal line represents the relationship of existing rates obtained by the model without addition of the evaluated response variable

(Supplementary Fig. 5) shows the classifications of haploids and diploids in maize leaves based on stomatal density.

According to the ROC curve, the model's discrimination capacity was 0.941, indicating that stomatal density is reliable to detect differences between haploid and diploid plants, which can assist in the disposal of diploid plants in early stages of culture. The obtained ROC curves for each hybrid are presented in Fig. 10.

Wu et al. (2013) subjected apical meristem of the diploid species *Clematis heracleifolia* tetraploidization using colchicine. Comparing leaves, the authors observed that leaves belonging to the tetraploid mutants had larger stomata than those of the leaves of diploid control plants. On average, tetraploid stomata were 16.7% greater in length, while diploid plants had stomata frequency around 8% higher. They suggested that the size and frequency of stomata are indirect indicators for an initial screening on plant ploidy. Likewise, Ribeiro (2022) reported that stomatal density varied according to source population, genotype and could serve as a potential discriminating trait between double-haploid and haploid plants.

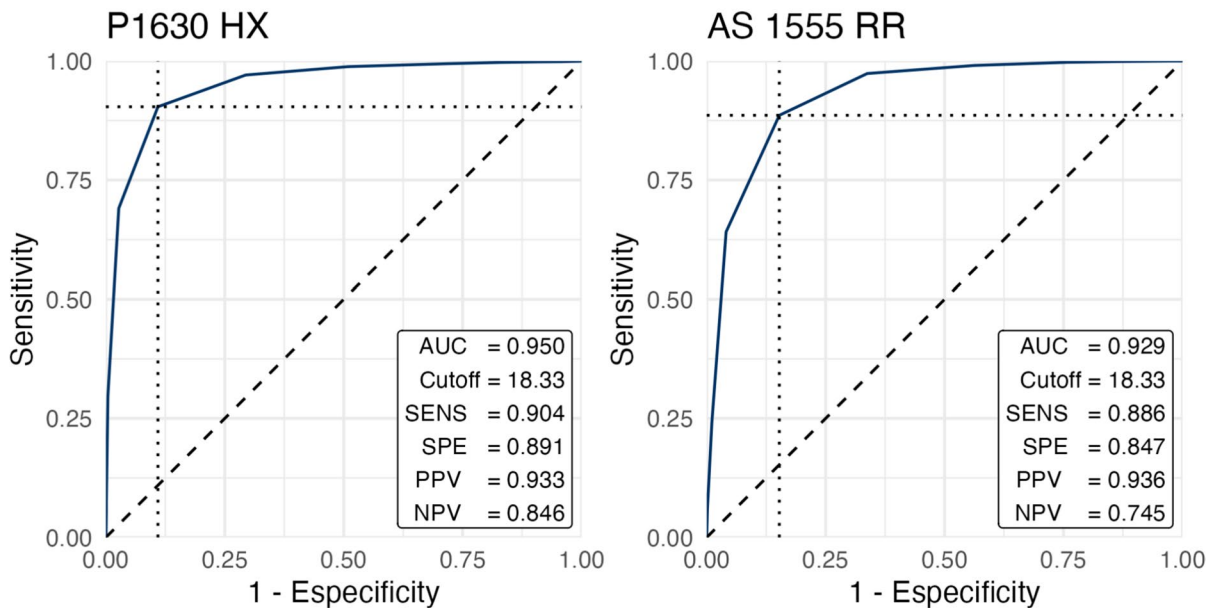
Table 8 Probability values associated with the cutoff point, SENS, SPE, PPV, NPV and AUC obtained for the classification of putative diploids and putative haploids, from the cutoff

point defined for the stomatal density (SD) in maize leaves, by means of the ROC curve

Variable	Probability	Cutoff Point (N/mm ²)	SENS (%)	SPE (%)	PPV (%)	NPV (%)	AUC
SD	0.505	18.33	89.4	87.2	93.5	80.1	0.941

SENS: sensitivity; SPE: specificity; PPV: positive predictive value; NPV: negative predictive value; AUC: area under curve.

Stomatal Density (SD) - ROC Curves

**Fig. 10** ROC curves and associated classification metrics for stomatal density (SD) in maize leaves, analyzed separately for the two hybrids

The findings of this study demonstrate that among the inducers tested, the (UH400×UH401)×TAIL 9 had the highest PHR when combined with five donor hybrids, indicating a possible accumulation of alleles responsible for haploid induction in this three-way cross inducer. For the two hybrids AS 1677 PRO3 and SYN 7205, inducer UH400×UH401 performed better. Together these results highlighted the differential performance of haploid inducers on putative haploid rate when used with various elite donor genotypes and endosperm textures. The findings also show that the guard cell length and the stomatal density were able to differentiate haploid and diploid plants in maize from two source genotypes using logistic regression. Because these traits were evaluated using only two donor

hybrids, future studies including a broader range of maize germplasm are necessary to confirm the general applicability of this method.

Acknowledgements To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná for the financial support.

Author Contributions Conceptualization: Marcondes MM, Faria MV, Scapim CA, Gomes R. Data curation: Faria MV, Scapim CA. Formal analysis: Rossi RM, Schuck ML. Methodology: Marcondes MM, Faria MV, Lima VA. Project administration: Faria MV. Writing-original draft: Schuck ML, Faria MV, Scapim CA. Writing-review & editing: Schuck ML, Faria MV, Scapim CA. All authors read and approved the final manuscript.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614). This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abdel CG, Stutzel H (2016) Stomata anatomy of 16 droughted and irrigated barley (*Hordeum vulgare*) genotypes. *J Univ Duhok* 19:146–156
- Beaulieu JM, Leitch IJ, Patel S, Pendharkar A, Knight CA (2008) Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytol* 179:975–986
- Bosabalidis AM, Kofidis G (2002) Comparative effects of drought stress on leaf anatomy of two olive cultivars. *Plant Sci* 163:375–379
- Chaikam V, Nair SK, Babu R, Martinez L, Tejomurtula J, Boddupalli PM (2014) 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
- Chaikam V, Martinez L, Melchinger AE, Schipprack W, Boddupalli PM (2016) Development and validation of Red Root Marker-Based haploid inducers in Maize. *Crop Sci* 56:1678–1688
- Chaikam V, Molenaar W, Melchinger AE, Boddupalli PM (2019) Doubled haploid technology for line development in maize: technical advances and prospects. *Theor Appl Genet* 132:3227–3243
- Chen YR, Lübberstedt T, Frei UK (2024) Development of doubled haploid inducer lines facilitates selection of superior haploid inducers in maize. *Front Plant Sci* 14:1320660
- Choe E, Carbonero CH, Mulvaney K, Rayburn AL, Mumm RH (2012) Improving in vivo maize doubled haploid production efficiency through early detection of false positives. *Plant Breed* 131:399–401
- Coe EH (1959) A line of Maize with high haploid frequency. *Am Nat* 93:381–382
- Couto EGO, Pinho ÉVRV, Pinho RGV, Veiga AD, Bustamante FO, Dias KOG (2015) In vivo haploid induction and efficiency of two chromosome duplication protocols in tropical maize. *Cienc Agrotecnol* 39:435–442
- 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
- De La Fuente GN, Frei UK, Trampe B, Nettleton D, Zhang W, Lübberstedt T (2018) A diallel analysis of a maize donor population response to in vivo maternal haploid induction: I. Inducibility. *Crop Sci* 58:1830–1837
- de O Pires RM, de RV Pinho ÉV, Souza GA, Vilela DR, dos Santos HO, Pereira WVS (2023) Anatomical tool for maize seedling ploidy identification and potential use in the double haploid obtainment process. *Euphytica* 219:93
- Demétrio CGB, Hinde J, Moral RA (2014) Models for overdispersed data in entomology. In: Ferreira C, Godoy W (eds) *Ecological modelling applied to entomology*. Springer International Publishing, Cham, pp 219–259
- Eder J, Chalyk S (2002) In vivo haploid induction in maize. *Theor Appl Genet* 104:703–708
- Franks PJ, Beerling DJ (2009) Maximum leaf conductance driven by CO₂ effects on stomatal size and density over geologic time. *Proc Natl Acad Sci U S A* 106:10343–10347
- Gilles LM, Khaled A, Laffaire J, Chaignon S, Gendrot G, Laplaige J, Bergès H, Beydon G, Bayle V, Barret P, Comadran J, Martinant JP, Rogowsky PM, Wdziez T (2017) Loss of pollen-specific phospholipase NOT LIKE DAD triggers gynogenesis in maize. *EMBO J* 36:707–717
- Gonçalves L, Subtil A, Oliveira MR, Bermudez PDZ (2014) ROC curve estimation: an overview. *Rev Stat J* 12:1–20
- Kelliher T, Starr D, Richbourg L, Chintamanani S, Delzer B, Nuccio ML, Green J, Chen Z, McCuiston J, Wang W, Liebler T, Bullock P, Martin B (2017) MATRILINEAL, a sperm-specific phospholipase, triggers maize haploid induction. *Nature* 542:105–109
- Khammona K, Dermail A, Suriharn K, Lübberstedt T, Wanchana S, Thunnom B, Poncheewin W, Toojinda T, Ruanchaichon V, Arikrit S (2024) Accelerating haploid induction rate and haploid validation through marker-assisted selection for qhirl and qhir8 in maize. *Front Plant Sci* 15:1337463
- Kim S, Rayburn AL, Lee DK (2010) Genome size and chromosome analyses in prairie cordgrass. *Crop Sci* 50:2277–2282
- Li L, Xu X, Jin W, Chen S (2009) Morphological and molecular evidences for DNA introgression in haploid

- induction via a high oil inducer CAUHOI in maize. *Planta* 230:367–376
- Liu Z, Wang Y, Ren J, Mei M, Frei UK, Trampe B, Lübberstedt T (2016) Maize doubled haploids. *Plant Breed Rev* 40:123–166
- Liu C, Li X, Meng D, Zhong Y, Chen C, Dong X, Xu X, Chen B, Li W, Li L, Tian X, Zhao H, Song W, Luo H, Zhang Q, Lai J, Jin W, Yan J, Chen S (2017) A 4-bp insertion at ZmPLA1 encoding a putative phospholipase a generates haploid induction in maize. *Mol Plant* 10:520–522
- Lomax BH, Woodward FI, Leitch IJ, Knight CA, Lake JA (2009) Genome size as a predictor of guard cell length in *Arabidopsis thaliana* is independent of environmental conditions. *New Phytol* 181:311–314
- Melchinger AE, Schipprack W, Würschum T, Chen S, Technow F (2013) Rapid and accurate identification of in vivo-induced haploid seeds based on oil content in maize. *Sci Rep* 3:2129
- Melchinger AE, Schipprack W, Friedrich UH, Mirdita V (2014) In vivo haploid induction in maize: identification of haploid seeds by their oil content. *Crop Sci* 54:1497–1504
- Melchinger AE, Molenaar WS, Mirdita V, Schipprack W (2016) Colchicine alternatives for chromosome doubling in maize haploids for doubled-haploid production. *Crop Sci* 56:559–569
- Mishra MK (1997) Stomatal characteristics at different ploidy levels in *Coffea* L. *Ann Bot* 80:689–692
- Molenaar WS, de Oliveira Couto EG, Piepho HP, Melchinger AE (2019) Early diagnosis of ploidy status in doubled haploid production of maize by stomata length and flow cytometry measurements. *Plant Breed* 138:266–276
- Moral RA, Hinde J, Demétrio CG (2017) Half-normal plots and overdispersed models in R: the hnp package. *J Stat Softw* 81:1–23
- Nanda DK, Chase SS (1966) An embryo marker for detecting monoploids of maize (*Zea mays* L.). *Crop Sci* 6:213–215
- Prasanna BM (2012) Doubled haploid (DH) technology in maize breeding: an overview. In: Prasanna BM, Chaikam V, Mahuku G (eds) *Doubled haploid technology in maize breeding: theory and practice*. CIMMYT, El Batán, pp 1–8
- Prigge V, Sánchez C, Dhillon BS, Schipprack W, Araus JL, Bänziger M, Melchinger AE (2011) Doubled Haploids in Tropical Maize: I. Effects of Inducers and Source Germplasm on in vivo Haploid Induction Rates. *Crop Sci* 51:1498–1506
- Prigge V, Xu X, Li L, Babu R, Chen S, Atlin G, 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
- Qu Y, Fernie AR, Liu J, Yan J (2024) Doubled haploid technology and synthetic apomixis: recent advances and applications in future crop breeding. *Mol Plant* 17:1005–1018
- R Core Team (2025) R: A language and environment for statistical computing. <https://www.R-project.org/>.
- Ren J, Zhang X, Li Z, Wu P (2022) Genetic analysis of maternal haploid inducibility for in vivo haploid induction in maize. *Agriculture* 12:845–845
- Ribeiro MR, Dos Santos TR, de Souza IRP, Carneiro AA, de Azevedo TC, Guimarães LJM, da Silva Chaves SF, Dos Santos Dias LA (2022) Are stomatal area and stomatal density reliable traits for identification of doubled haploids in maize? *Crop Breeding and Applied Biotechnology* 22:e42222226
- Rober 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
- Silva HA, Scapim CA, Vivas JMS, Amaral Júnior AT, Pinto RJB, Mourão KSM, Rossi RM, Baleroni AG (2019) Effect of ploidy level on guard cell length and use of stomata to discard diploids among putative haploids in maize. *Crop Sci* 60:1199–1209
- Silva HA, Scapim CA, Vivas M, Amaral Júnior AT, Pinto RJB, Vivas JMS, Rossi RM, Baleroni AG (2020) Corn genotypes and crop seasons on the rate of putative haploids with the expression of gene *R1-navajo*. *Pesq Agrop Brasileira* 55:e01521
- Silva JPA, Viana JMS, Dias KOG, Silva JC, Tupper VT, Clarindo WR (2023) Popcorn (*Zea mays* L. var. Everta) haploids identified by Navajo phenotype and ploidy level. *Front Plant Sci* 14:1176504
- Trentin HU, Bafrú G, Frei UK, Dutta S, Lübberstedt T (2022) Investigating the effect of the interaction of maize inducer and donor backgrounds on haploid induction rates. *Plants* 11:1527–1527
- Trentin HU, Krause MD, Zunjare RU, Almeida VC, Peterlini E, Rotarencu V, Frei UK, Beavis WD, Lübberstedt T (2023) Genetic basis of maize maternal haploid induction beyond MATRILINEAL and ZmDMP. *Front Plant Sci* 14:1218042
- Wang H, Dong B, Jiang J, Fang W, Guan Z, Liao Y, Chen S, Chen F (2014) Characterization of in vitro haploid and doubled haploid *Chrysanthemum morifolium* plants via unfertilized ovule culture for phenotypical traits and DNA methylation pattern. *Front Plant Sci* 5:0738
- Wu Y, Li W, Dong J, Yang N, Zhao X, Yang W (2013) Tetraploid induction and cytogenetic characterization for *Clematis heracleifolia*. *Caryologia* 66:215–220
- Xu Z, Zhou G (2008) Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *J Exp Bot* 59:3317–3325
- Zhao W, Sun Y, Kjellgren R, Liu X (2015) Response of stomatal density and bound gas exchange in leaves of maize to soil water deficit. *Acta Physiol Plant* 37:1704
- Zhong Y, Liu C, Qi X, Jiao Y, Wang D, Wang Y, Liu Z, Chen C, Chen B, Tian X, Li J, Chen M, Dong X, Xu X, Li L, Li W, Liu W, Jin W, Lai J, Chen S (2019) Mutation of ZmDMP enhances haploid induction in maize. *Nat Plants* 5:575–580

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.