ARTICLE

Are stomatal area and stomatal density reliable traits for identification of doubled haploids in maize?

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Abstract: *To meet the demands of the production chain, breeding programs need to reduce the time for selection and development of higher yielding maize genotypes. This involves application of new strategies, such as the use of the doubled haploid lines. We evaluated the use of stomatal area and density in distinguishing doubled haploids (DH) from false positive (FP) plants derived from nine tropical source populations. We selected four DH plants and two FP plants for identification of DH through leaf anatomy in the populations, in each group. Data on stomatal area and density were considered in a completely randomized model in a factorial scheme (populations and genotypes) and analyzed by restricted maximum likelihood. There were statistically significant effects of populations and genotypes for both traits. For stomatal density, the population by genotype interaction was not verified. Stomatal density proved to be efficient for distinguishing DH and FP for use in selection of doubled haploids.*
Keywords: Zea mays*, leaf anatomy, doubled haploid technology, false positive*

INTRODUCTION

Maize is the main cereal crop grown worldwide and, together with soybean, constitute the two main agricultural products in Brazil. Brazilian maize production in the 2021/22 season is estimated at 114 million metric tons. The country is the third largest producer worldwide and the second largest exporter of the crop. World production will reach 1.2 billion tons in 2021/22 (USDA 2022). The large production of maize in Brazil shows its importance in human and animal nutrition and as an input in industry, which leads to greater demand and, possibly, larger planted area.

To meet the demands of the production chain, plant breeding programs need to reduce the time for selection and development of higher yielding maize genotypes that are resistant to stresses and have traits that facilitate management. This involves application of new strategies, such as the use of the technology of doubled haploid (DH) lines (Xu et al. 2017, Chaikam et al. 2019). The aim of the DH technology is to reduce the time necessary for obtaining homozygous lines by one and a half year (three generations), allowing uniform and genetically stable lines to be acquired. This advantage of the DH technology

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has led seed companies to use it on a broad scale to reduce time and costs in releasing hybrids (Liu et al. 2016). Thus, DH technology has become a focus of research in companies and universities (Couto et al. 2013).

Visual analysis is the first parameter used in the identification of haploid embryos. It is based on the phenotypic marker expressed by the gene *R1-navajo* (*R1-nj*), which codifies the anthocyanin pigmentation of the endosperm and embryo of maize seeds (Nanda and Chase 1966). Visual analysis allows selection of seeds that will give rise to possible haploids from the embryos that are white, having genes only from the source genotype (Prasanna et al. 2012).

In spite of its efficiency for initial selection of haploids, visual analysis of seeds is subject to inaccuracy in the process of identification. Such inaccuracy is due to the *R1-nj* marker, which can be completely inhibited or segregate in the grain in the cross of haploidy induction, due to the existence of alleles that interfere in the anthocyanin synthesis pathway, which may occur with greater frequency in populations of tropical origin (Chaikam et al. 2015). Thus, it is necessary to evaluate traits that allow later identification and exclusion of false positives (FP), that is, distinguishing plants whose seeds were erroneously identified as haploids (through errors in selection or inhibition in the formation of anthocyanin in the embryo), from putative haploids, which would be the true haploid individuals, correctly identified, promoting reduced expenditures on inputs, labor, and space (Choe et al. 2012, Souza et al. 2018).

Various traits have been analyzed for selection of haploids in maize, such as the color of seminal roots (Prassana et al. 2012, Chaikam et al. 2016), oil content in the embryo (Melchinger et al. 2014), seed weight (Smelser et al. 2015), seedling vigor (Ribeiro et al. 2018), and length of the shoot and roots (Chaikam et al. 2017). Often, analytical procedures such as flow cytometry (Couto et al. 2013, Han et al. 2016, Ribeiro et al. 2018) and anatomical analysis of the stomata (Choe et al. 2012, Souza et al. 2018) are also used. These methods make it possible to confirm the haploidy of plants in the seed or seedling stages and eliminate diploid plants that were not excluded by the previous parameters.

Anatomical analysis to identify haploid plants is an effective methodology and can be performed in the initial phases of plant development, with rapid results at low-cost (Souza et al. 2018). Analyzing the length of stomatal guard cells is a methodology used for discrimination of ploidy, because it has been correlated with DNA content in various species. Changes in the level of ploidy in plants result in changes in the size and anatomy of their organs and cells, and such changes can be identified by microscopy. In the *Clematis heracleifolia* chromosomal duplication with a colchicine solution generated individuals with greater stomatal length and width (Wu et al. 2013). In *in vitro* chromosomal duplication of unfertilized ovules of *Chrysanthemum sp*., greater stomatal length was observed in doubled haploids than in individual haploids (Wang et al. 2014). Furthermore, crossing the male-sterile wheat line (*Triticum sp.*) with the haploid inducer maize genotype generated haploid plants that had guard cells that were longer than the guard cells of diploid control plants (Zhang et al. 2014).

The aim of the study was to evaluate the efficiency of use of stomatal area and density in distinguishing DH from FP plants derived from tropical source populations of maize, increasing the efficiency for obtaining and selecting DH lines.

MATERIAL AND METHODS

The trial and its evaluations

The trial was conducted at Embrapa Milho e Sorgo, in Sete Lagoas, Minas Gerais, Brazil, from December 2017 to July 2019. We evaluated DH and FP lines derived from nine source populations, obtained from biparental crosses between elite lines of the breeding program of Embrapa Milho e Sorgo, being five source populations from the Flint heterotic group and four from the Dent group. The F_1 crosses were made only between lines of the same heterotic group. After that, the F_1 source populations were self-fertilized, resulting in the F_2 generation. Then the lines of the F_2 generation were self-fertilized to obtain the F_3 generation. Finally, haploidy was induced in an isolated lot for each source population (F_1, F_2, and F_3). However, haploids were not obtained from all the generations, due to losses in the field, resulting in 16 populations of DH lines (Table 1).

The field for haploidy induction was set up in December 2017 by sowing the nine source populations in individual rows interspersed with the haploid gynogenetic inducer hybrid TAIL P1 × TAIL P2, in a 1:1 proportion. The haploidy inducer field was isolated by 1000 m from other maize crops in the vicinity. Seeds were mechanically sown in 4.2-m-long rows

and between-row spacing of 0.70 m. The crop treatments in the induction field were performed as recommended by Amaral Filho et al. (2005). At anthesis, all the plants of the source populations had been detasseled so as to allow only the pollen derived from the haploidy inducers to be in the field.

Manual harvest of the field was carried out in April 2018, and ears from each source population were placed in bags with tags identifying origin and heterotic group. Seeds were selected through the *R1-navajo* (*R1-nj*) marker. In this system, the seeds of each ear that had purple pigmentation in the endosperm and absence of pigmentation in the embryo were selected as haploid seeds. After identification of haploids, chromosomal duplication was carried out to obtain doubled haploid lines.

Table 1. Doubled haploid lines evaluated in the experiment, their generations (F_1, F_2, F_3) and heterotic groups

Nr. of source populations (P)	Generations	Heterotic groups
1	F_1, F_3	Flint
2	F_3	Flint
3	F_2, F_3	Flint
4	F_2, F_3	Flint
5	F_1, F_2	Flint
6	F_1	Dent
7	F_1, F_2, F_3	Dent
8	F_1, F_3	Dent
9	F_3	Dent

For chromosomal duplication, 50 seeds from each source population were separated, resulting in nine groups and a total of 800 plants (16 populations x 50 plants). Chromosomal duplication was carried out in January 2019, adopting the protocol of Couto et al. (2013). The haploid seeds were sown in plastic trays containing vermiculite and kept in greenhouse at a temperature of 25 ºC and relative humidity of 60%. At 15 days after sowing, the seedlings went through the chromosomal duplication process in the doubled haploid technology laboratory of Embrapa Milho e Sorgo. Initially, the seedlings were carefully removed from the trays, and the roots were washed in running water. They were then grouped in bundles of 25 seedlings, identified according to their source population and each bundle was inserted in 800-mL beakers with water, to avoid wilting. For chromosomal duplication, a solution was prepared composed of the following reagents: 1% colchicine (w/v), 1% dimethyl sulfoxide (v/v), and 1% Tween 20 (v/v). After preparation, 185 mL of this solution was distributed in 600-mL beakers. These beakers were transferred to a fume hood, and 25 identified seedlings was inserted in each one. The seedlings were kept under treatment for six hours in the absence of light and at ambient temperature.

After six hours of treatment in the colchicine solution, the seedlings were removed from the solution and placed in cube-shaped screened containers, and then transferred to a tank for spray washing for 30 minutes. After that, the supposed doubled haploid seedlings were transplanted to plastic trays containing commercial substrate and transported to a greenhouse, where they were kept for 20 days for recovery from the colchicine treatment. The supposed doubled haploid seedlings were carefully removed from the trays with substrates and manually transplanted to 20-L pots with soil fertilized using a dose equivalent to 2000 kg ha^{-1} of 4-28-16 (N-P-K) + Zn. Three seedlings were transplanted per pot and identified by treatment in February of 2019, and the pots were kept in a climate-controlled greenhouse. Management practices after planting were carried out according to crop requirements (Amaral Filho et al. 2005).

For identification of DH through leaf anatomy, six genotypes were selected in the V6 stage (four DH and two FP plants) in each one of the nine groups and generations of source populations (Table 1). DH and FP were differentiated based on morpho-agronomic traits (plant height, leaf angle, and stem and leaf color). A sample from the fifth fully expanded leaf (counting from lower to higher) was collected from each plant, resulting in a total of 96 samples (6 plants x 16 populations), considering the nine groups of DH lines. To standardize collection of samples in the plants selected, on the fifth leaf of each plant, a measurement of 3 cm was made in the direction from the base to the tip. Starting at this point, a 1 cm rectangular sample of leaf was collected and cut in a direction transversal to the middle part of the leaf blade. These samples were placed in labeled tubes containing 70% ethanol. The tubes were then taken to totally remove chlorophyll from the leaf and obtain estimates of stomatal size and density. Removal was facilitated by four exchanges of 70% ethanol at intervals of 24 hours over three days.

To capture images of the stomata, a rectangular subsample was collected from the leaf sample after removal of chlorophyll, using a scalpel. After that, 70% alcohol was added to the glass slide, covering the sample with a cover slip. Microscopic analyses of the slides were carried out without the use of staining. The stomata were visualized using a stereoscopic electronic microscope, at maximum magnification (112 x), with an attached camera at the magnification

of 112 x 0.459 mm. The lens was focused on the region near the midrib on the abaxial part of the leaf, and the image was transmitted to a computer for measurement. To obtain the size of the stomata, a fixed size scale of the equipment was added for assessment, measuring the height and width of the extremities of the guard cells of each stoma, with a total of 10 stomata/image, for each one of the six genotypes selected (four DH and two FP plants). After that, the area of each stoma was estimated through application of the formula:

$$A = a.b.\pi$$

where a is the smaller radius and b is the larger radius. In the same area in which the stomata were measured, stomatal density was evaluated by manual count in a standard image area of 112 x 0.459 mm.

After self-pollination of the plants and harvest, preliminary selection of the DH sampled was confirmed by morpho-agronomic traits. Thus, ears with one or few seeds of yellow color confirm a DH plant, whereas ears with high grain formation and seeds with white color and/or the presence of anthocyanin indicate FP. This information was crossed with the data on stomatal density and area obtained in the evaluations for identification of putative DH and FP.

The statistical procedures

First of all, it is necessary to bear in mind that haploids were not obtained from all the generations (F_1, F_2, and F_3) due to loss of plants/generations, e.g. only population 7 had the three generations (see Table 1). This situation resulted in an unbalanced dataset. For this reason, it was decided to process the data by residual maximum likelihood (Patterson and Thompson 1971), a suitable method for dealing with unbalanced data. In addition, the generation factor was disregarded, assuming that all populations were in the same generation. In terms of breeding, the progenies of the crosses of the different groups of population, in different levels of inbreeding (generations), when pollinated exclusively by the inducing single hybrid TAIL P1 x TAIL P2, have inbreeding coefficients equal to zero, regardless of the plants used as mothers and their respective previous levels of inbreeding.

The statistical model used to determine the stomatal area and density data, considering a completely randomized design in a factorial scheme, was:

$$y = \mu + p_i + g_j + pg_{ij} + \varepsilon_{ij}$$

where y is the phenotypic observation, μ is the intercept, p_i is the effect of the i-th population, g_j is the effect of the j-th genotype (DH or FP - false positive), pg_{ij} is the effect of the interaction of the i-th population with the j-th genotype and ε_{ij} is the residual effect. All model effects were considered fixed, with the exception of the residual.

The significance of the effects was verified using the Wald test (Wald 1945) and the adjusted means for the fixed effects were obtained by the BLUE (Best Linear Unbiased Estimator) method (Henderson 1975). The decision on whether the adjusted means differed was based on confidence intervals, obtained by:

$$Lim = \text{BLUE} \pm 1.96 * \text{SE}$$

where Lim is the upper or lower limit and SE is the standard error of the BLUE estimate. All analyses were performed in the R software (R Core Team 2022), using the ASReml-R package (Butler et al. 2018).

RESULTS AND DISCUSSION

For both stomatal area (µm²) and density (number of stomata per area of 112 x 0.459 mm) the Wald test value indicated significance for the effects of populations and genotypes (Table 2). The difference between genotypes (DH and FP) corroborated the possibility of using both stomatal area and density as differentiators between haploids and diploids (Choe et al. 2012, Souza et al. 2018). There was a highly significant effect of the interaction between genotypes (DH and FP) and populations on stomatal area. In other words, genotypes react differently depending on the source population from which they are obtained, when considering the stomatal area. On the other hand, this dependence was not verified for stomatal density (Table 2), indicating the potential of this trait to distinguish DH from FP.

The experimental coefficients of variation (CV) were similar for both stomatal area and density (approximately 27%). These CV values were high for laboratory experiments where environmental conditions are controlled. Souza et

Table 2. Summary of the statistical analyses for stomatal area and density, evaluated in double haploid lines and false positives (Genotypes) from nine source populations

	Stomatal area (μm^2)		**Stomatal density** (stomata per area of 112 x 0.459 mm)	
Sources of variation	**df**	**Wald test value**	**df**	**Wald test value**
Population (P)	8	66.50 **	8	31.97 **
Genotype (G)	1	97.40 **	1	22.53 **
P x G	8	174.6 **	8	13.27
Error	692		73	
Mean		821.45		46.12
Minimum		596.08		28.75
Maximum		1317.78		69.25
CV (%)		26.74		27.04

** Significant effects at 1% probability.

al. (2018) evaluated doubled haploid, haploid and diploid progenies, and found CV of 12.33% for the stomatal density and 10.41% for the guard cell length. However, these authors evaluated only two progenies for each type of genotype, whereas, in our experiment, nine progenies coming from different source populations were evaluated. This resulted in greater variability in the genotypes under assessment, with wide amplitude detected for stomatal area (596 to 1318 μm^2) and stomatal density (29 to 69 stomata per area of 112 x 0.459 mm) (Table 2).

Stomatal density and stomatal area are traits arising from leaf anatomy. Several studies attest to the utility of leaf anatomy as a tool for assisting the selection of genotypes (DH and FP plants). Choe et al. (2012) evaluated haploid, double haploid, false positive and diploid maize and found that the guard cell length from the fifth leaf allowed the separation between these four classes with greater precision. Souza et al. (2018) concluded that measuring the traits of stomatal guard cell length and stomatal density would be an efficient and non-destructive methodology for selecting DH and FP plants. Stomatal density as a differentiating trait of haploids, compared to stomatal area and guard cell length, can be implemented using simple observation and counting under microscopes, adding greater ease of measurement.

Thus, the implementation of the leaf anatomy as a tool to assist in the selection of haploids and doubled haploids can increase efficiency in the production of DHs, allowing the differentiation of diploids (FP) from haploid and doubled haploids at an initial stage. Haploidy may be induced in any type of population and generation. In maize, the technology of DH lines has been used in obtaining lines from F_1 hybrids (Trindade et al. 2019), selected according to the aims of the program. Depending on the genetic background of the germplasm used, mainly in tropical germplasm, identification in seeds can become difficult, due to the inhibition of the marker R1-nj. This fact makes a secondary trait, such as stomatal density, advantageous for differentiating haploids.

In terms of genotype, for the stomatal density, the Flint group seemed to exhibit greater variability (Figure 1A). For stomatal area the variability was greater for the Dent group (Figure 1B). The data obtained corroborate that the values of stomatal area and number of stomata are affected by the genotype (Table 2). However, the type of genotype can be a differentiation factor. In relation to stomatal density, DH lines were distinguished statistically from FP lines (Figure 2). On the other hand, stomatal area was more reduced in DH lines than in diploid individuals (FP) (Figure 2), denoting a compensatory effect, in which the smaller stomatal area is balanced by the greater density of the stomata per leaf (Figure 2).

The tendency to a larger number of stomata with smaller area in the DHs evaluated is also noteworthy, indicating an effect directly associated with photosynthetic processes (Melkonian et al. 2004). This denotes that smaller stomatal area, which would hinder gas exchanges, is compensated by greater stomatal density. Souza et al. (2018) evaluated the types of genotypes (haploids, doubled haploids, and diploids) and found that the diploids, false positives, formed a distinct group from the haploids and doubled haploids. The same researchers emphasized that the diploids exhibited intermediate values for stomatal density.

Leaf anatomy has been the focus of studies for different species. Through this methodology it is possible to associate the size of stomatal guard cells and ploidy levels. A study with sweet peppers (*Capsicum annuum* L.) confirmed that the mean stomatal length was greater in diploids (35.2 ± 2.5 µm) and smaller in haploids (26.4 ± 2.4 µm) (Shrestha and Kang 2016). Przywara et al. (1988) studied kiwi (*Actinidia deliciosa*) and found correlation between the length of the stomatal guard cells and distinction of haploid and diploid plants (24 ± 1.7 µm and 33 ± 2.4 µm, respectively). Choe et al. (2012) found that the mean length of the stomatal guard cells is an efficient trait for differentiation of haploid and diploid plants in maize. Sekiya et al. (2020) evaluated samples of maize leaves collected in the V2/V3 stages and found that the morphometry of the stomata is an efficient methodology for distinction between haploids and false positives in sweet corn. Thus, our study confirms the efficiency of evaluation of stomatal density for distinction of DH and FP and for selection of DH.

FINAL CONSIDERATIONS

For the set of DH evaluated, stomatal area showed variation as a function of the source population, genotype and the interaction between both factors, making it difficult to use it as a trait to distinguish between DH and FP.

In turn, stomatal density varied as a function of source population and genotype, but showed no interaction between these factors, which could serve as a differentiating trait between DH and FP.

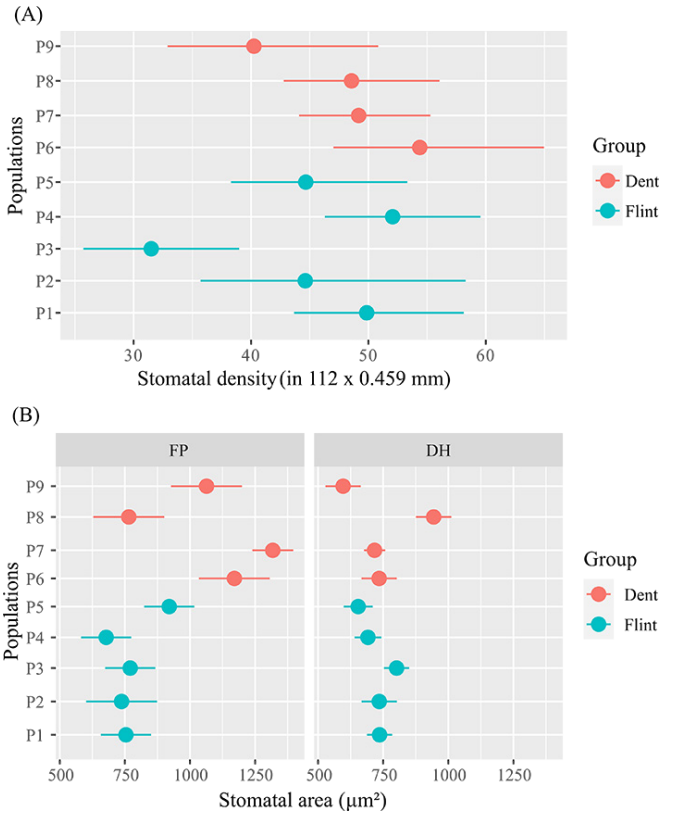

Figure 1. BLUES of stomatal density (A) and area (B) (with bars showing the confidence interval) of nine source populations of Flint and Dent groups, considering DH (doubled haploid) and FP (false positive) genotypes.

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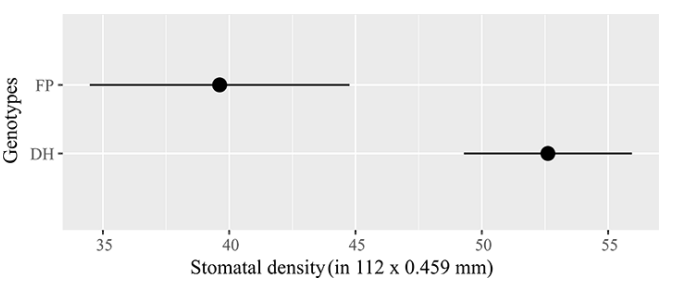

Figure 2. BLUES of stomatal density (with bars showing the confidence interval) of DH (doubled haploid) and FP (false positive) genotypes.

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