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ARTICLE

Early haploid identification by stomatal guard cell length in tropical supersweet corn using different inducers

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Abstract: *Identification of early haploids is necessary to increase the efficiency of the double haploid production. The objectives were to determine the effectiveness of early selection of haploid seedling and to investigate the haploid inducers and donor tropical supersweet corn interactions, based on stomatal guard cell length. Two haploid inducers, three supersweet corn populations and its six combinations of F_1's diploid and haploid, classified based on the expression of the R1-navajo gene and by the colors of the first leaf sheath in seedlings at the V2 stage, were used. From each seedling of the treatments, external epidermal impressions were taken to measure the stomatal guard cell length. F_1 haploids showed a 32.7% to 38.2% reduction in guard cell length compared to F_1 diploids. The parents contributed differently to reduce or increase guard cell length in the F_1's combinations, but without specific interactions between parents.*

Keyword: Zea mays var. saccharata*, double-haploid, haploid selection, R1-nj gene, diploids*

INTRODUCTION

Supersweet corn (*Zea mays* var. *saccharata*) is classified as a special type that results from the natural mutation of common corn alleles and is considered a vegetable intended for human consumption (Pereira Filho and Teixeira 2016). In Brazil, this crop is aimed at the canning industry, which demands cultivars with high yield potential, quality, and uniformity, traits commonly found in hybrids (Teixeira et al. 2013).

Development of inbred lines is an essential part of hybrid breeding programs. The conventional process of obtaining inbred lines is time-consuming and costly, as it involves around six to eight generations of self-pollination (Prigge et al. 2011). The double-haploid technology has been widely used to accelerate this process of obtaining lines in common corn, producing completely homozygous lines in a total of two to three generations. This approach consists of four steps: haploid induction, haploid identification, chromosome doubling, and seed production from double haploid lines (Chaikam et al. 2019). Despite the advantages of using double haploids in common corn, there are some studies on induction rates in

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supersweet corn (Sekiya et al. 2020, Silva et al. 2020, Trentin et al. 2022), but few reports exist on their effective use to generate inbred lines (Khulbe et al. 2020).

Effective methods of identifying haploids at the seed or seedling stages are essential to increase the efficiency of obtaining double haploid lines (Chaikam et al. 2016). The best-known means of identifying haploids is the use of the *R1-navajo* (*R1-nj*) dominant gene, which expresses anthocyanin pigmentation in seeds, helping to identify haploids and diploids (Silva et al. 2020). Thus, haploid seeds resulting from induction crosses are identifiable by a purple endosperm and no purple embryo (Prigge et al. 2012a). However, the expression of this marker may show incomplete penetrance and variable expressivity depending on the germplasm source, inducing material, and environmental factors (Kebede et al. 2011), resulting in failures in haploid classification, which reduces the proportion of true DH lines produced (Silva et al. 2020). Studies have shown that part of the tropical germplasm has genes that inhibit the expression of the *R1-nj* gene, such as the *Cl-1* (Kebede et al. 2011, Prigge et al. 2012b, Chaikam et al. 2015). Thus, the identification of haploids by nondestructive methods can contribute to higher efficiency in obtaining DHs, improving logistics and reducing the waste of resources used in the chromosomal doubling stage (Choe et al. 2012).

These failures in the process of correctly identifying haploids based on seed color and the occurrence of *R1-nj* gene inhibition have revealed a need for complementary strategies to increase the efficiency of haploid identification, e.g., inducers with high seed oil content (Melchinger et al. 2014, Wang et al. 2016), root color marker (Chaikam et al. 2016), transgenic markers such as green fluorescent protein (GFP) (Yu and Birchler 2016), double fluorescent proteins (eGFP and dsRED) (Dong et al. 2018) and RUBY reporter (Wang et al. 2023), but all of these are presented only in specific inducers with restrict access and are unavailable for tropical environments.

Other strategies such as flow cytometry (Couto et al. 2013, Baleroni et al., 2021), chromosome counts (Sekiya et al. 2020), and molecular markers (Ribeiro et al. 2018) can be used, but are less efficient, expensive and laborious. Therefore, the use of morphological traits of seedlings and plants (Chaikam et al. 2017), such as first-leaf-sheath color (Sekiya et al. 2020) associated with stomatal guard cell length or area (Choe et al. 2012, Molenaar et al. 2019, Ribeiro et al. 2022), are important alternatives for haploid identification in corn. The green first-leaf-sheath is a recessive characteristic and can be observed in some corn genotypes, such as the supersweet genotypes studied by Sekiya et al. (2020), which allows 100% accuracy in the early haploid seedling selection, as the diploid inducers and F1's have a purple first-leaf-sheath. However, most tropical germplasm also has purple leaf sheaths, limiting its use in haploid identification.

Studies reveal that diploids and haploids can be efficiently distinguished in two- or three-leaf stage, based on stomatal guard cell length (Choe et al. 2012, Molenaar et al. 2019, Sekiya et al. 2020, Silva et al. 2020), due to lower DNA content in haploids compared to diploid cells (Lomax et al. 2009). This technique is especially important in tropical genotypes with presence of genes that inhibit the expression of the *R1-nj* (Chaikam et al. 2015, Chaikam et al. 2016) Although the reduction in guard cell length in haploid F1's is known, compared to their respective diploid F_1's, it is essential to identify if different inducers would produce the same reduction effects in crosses with different donor populations, and whether these effects are differentiated according to specific combinations of parents, in order to allow the identification of a standard index of reduction for the identification of haploid seedlings in the V2 stage.

The objectives of this study were to determine the effectiveness of early selection of haploid seedling and to investigate the haploid inducers and donor tropical supersweet corn interactions, based on stomatal guard cell length.

MATERIAL AND METHODS

In the 2019/2020 growing season, three supersweet corn populations were pollinated individually by two haploid inducer populations, on the Fazenda Escola (lat 23º22' S, long 51º33' W, alt 576 m asl), State University of Londrina (UEL), in the State of Paraná, Brazil, to obtain the respective F1 generations. The three tropical populations of supersweet corn (SD3004, SD3005, and SD3006), were developed by the Maize Breeding Program of State University of Londrina, with the introduction of the *shrunken* gene (*sh2*) in tropical maize populations that show green first-leaf-sheath. The two gymnogenetic haploid inducers (PI4001 and PI4003) were derived from crosses of ancient Stock 6 with two different adapted tropical populations.

The seeds of the F_1 generations were classified visually, based on the expression of the *R1-nj* gene, into diploid F_1's (seeds with purple endosperm and embryo) and haploid F_1's (seeds with purple endosperm and embryo not purple).

During the 2020/2021 growing season, in a greenhouse at the UEL campus, samples of seeds of the five parents, the six diploid F_1's and the six haploid F_1's combinations were sown in plastic trays with 64 cells, which were filled with Sphagnum peat.

At the V2 stage, the seedlings obtained from the 17 treatments were evaluated based on stomatal guard cell length (SGCL). Epidermal impressions were collected from 1 cm long leaf samples (Figures 1a, b), which were sectioned

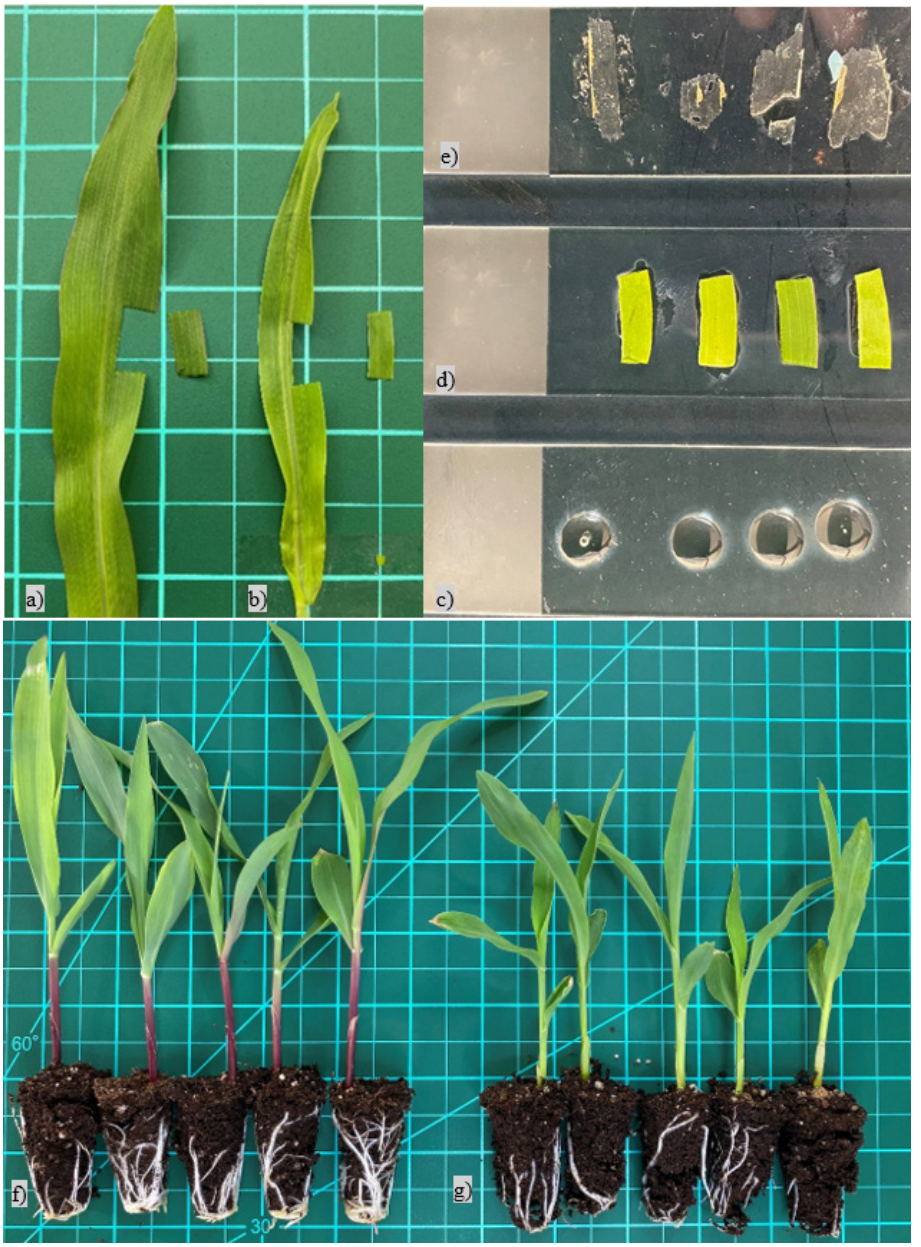

Figure 1. Steps to collect leaf epidermal impressions in diploid and haploid seedlings: a) diploid leaf samples cut; b) haploid leaf samples cut; c) drops of superglue; d) leaf sample placed over the superglue; e) leaf epidermal impression; f) diploid seedlings; g) haploid seedlings.

transversely, from the mid portion of the blade of the second leaf and placed individually on a drop of superglue on microscope slides to evaluate the abaxial face (Figures 1c, d), following the methodology of Choe et al. (2012). After the glue had dried, the plant tissue was carefully removed (Figure 1e) and the images of the impressions left by the stomatal guard cells on the microscope slides were captured using a Floid Cell Imaging Station microscope (Thermo Fisher Scientific, Waltham, MA, USA) at 460x magnification (Figure 2). Photographic images were taken from each sample to measure the length of six normal and representative guard cells. A total of 120 to 480 guard cells were evaluated per treatment, depending on the number of haploid plants obtained. Measurements were performed using MicroMeasure software version 3.0 (Colorado State University, Fort Collins, CO, USA), with a standardized scale.

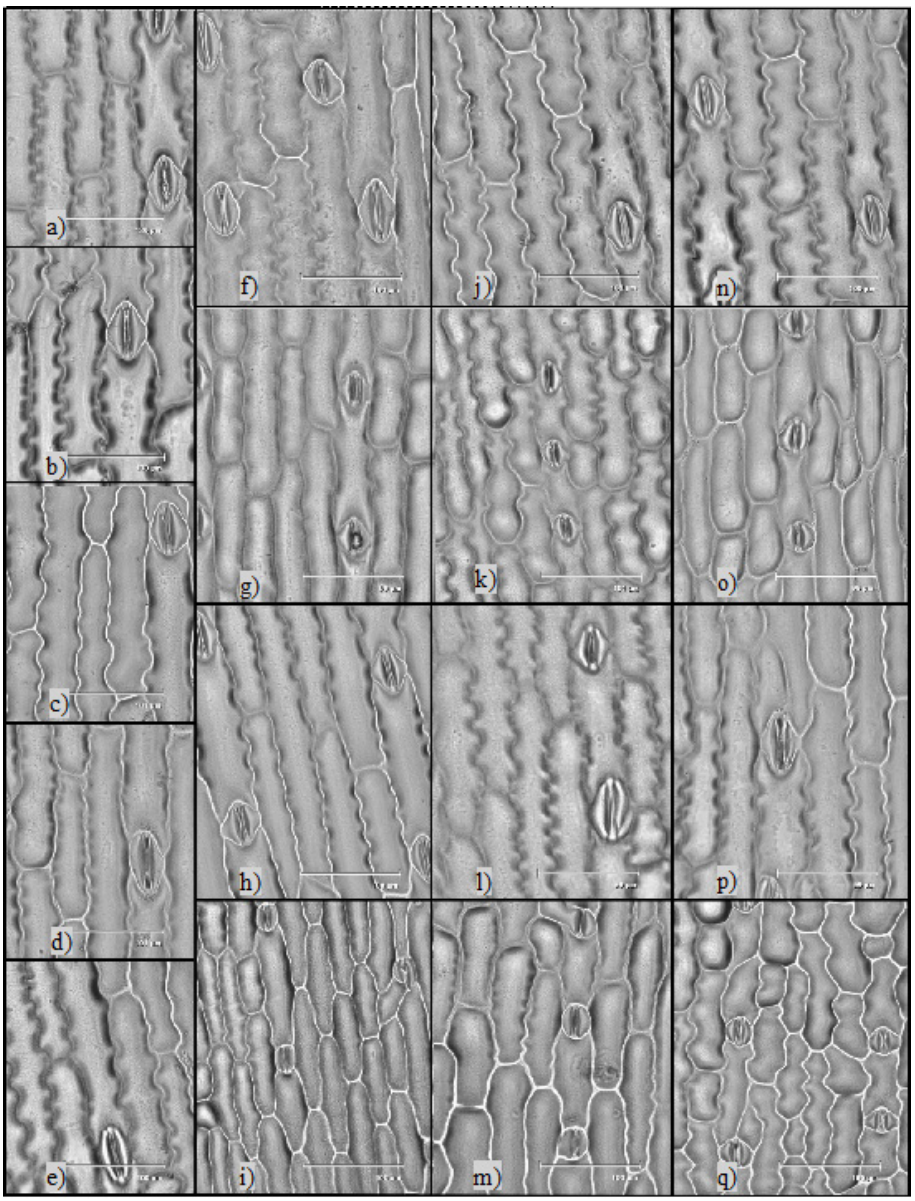

Figure 2. Microscope slides of the leaf epidermal impressions; a) inductor PI4001 (I1) ; b) inductor PI4003 (I3); c) population SD3004 (P4); d) population SD3005 (P5); e) population SD3006 (P6); f) diploid F1 - P4×I1; g) haploid F1 - P4×I1; h) diploid F1 - P4×I3; i) haploid F1 - P4×I3; j) diploid F1 - P5×I1; k) haploid F1 – P5×I1; l) diploid F1 - P5×I3; m) haploid F1 – P5×I3; n) diploid F1 – P6×I1; o) haploid F1 – P6×I1; p) diploid F1 – P6×I3; q) haploid F1 – P6×I3;

Among the seedlings of the six haploid F_1's, all of those with the first leaf sheath purple were eliminated as they were false-haploid, as found by Sekiya et al. (2020), who used these same three populations and confirmed, through chromosome counting, that the haploids had a green first leaf sheath (Figure 1g) and the diploids had a purple one (Figure 1f).

The treatments were evaluated in a completely randomized design in which each seedling was considered a repetition. Six measurements of stomatal guard cell length were taken per seedling.

The following linear random model was used in the analysis of variance: $Y_{ijk} = \mu + t_i + e_{ij} + w_{k(ij)}$, where: Y_{ijk} = is the observed value to ith treatment of the jth repetition in the kth stomatal guard cell; μ is the fixed overall mean; t_i = is the fixed effect of ith treatment (i=1,..., I); e_{ij} = is the random effect of the jth repetition (j=1,..., J); $w_{k(ij)}$ = is the random effect of the kth stomatal guard cell within ith treatment in the jth repetition (k=1,..., K). The degrees of freedom of treatment were decomposed in parents *per se*, diploid and haploid F_1 generations, and two means contrasts (vs.): a) (Parents and Diploid F_1s) vs. haploid F_1s; b) Parents vs. Diploid F_1s. The Anova and comparison of means using Tukey's test were performed with the GLM procedure of the SAS software, version 9.0 (SAS Institute Inc., Cary, NC, USA).

Based on the average of six SGCL measurements per seedling, a frequency distribution study was carried out for the sets of treatments with diploid and haploid individuals to identify the occurrence of overlapping-distribution graphs at different ploidy levels.

RESULTS AND DISCUSSION

The seeds obtained from the crossing of inducing populations and tropical supersweet populations were marked by the expression of the *R1-nj* gene, which allowed the classification of F1 seeds into diploids and putative haploids. In this case, the tropical supersweet corn populations did not have genes that inhibit the expression of the *R1-nj* gene, as cited by Chaikam et al. (2015).

At the V2 stage, all seedlings from the seeds of inducers and the diploid F1 generations showed a purple first leaf sheath phenotype (Figure 1f), whereas the seedlings originating from the populations of supersweet corn exhibited a green color (Figure 1g). However, the seeds of the F1 generations, early classified as haploid, originated on average 64% of seedlings with a green leaf sheath and 36% purple, which were discarded and considered false haploids as they had the dominant allele of the inducers that expresses the purple color in the first leaf sheath, as shown by Sekiya et al. (2020).

Just as the homozygous recessive genotypes for the *liguleless* gene, which conditions the absence of a ligule in the leaves, used in the early confirmation of true haploids for the breeding of inducers (Melchinger et al. 2016), these supersweet corn genotypes without genes that inhibit anthocyanin expression and with the first leaf sheath green are an efficient alternative to assist in the early assessment of the true rates of haploid induction and in the breeding of inducers.

Analysis of variance among and within replicates for SGCL indicated higher variance among plants used as repetition in the treatments (13.29 μm^2) than within plants (7.08 μm^2), as estimated from the measurements of six SGCL per plant (Table 1). These results suggest that the measurement of a representative SGCL within

Table 1. Analysis of variance with decomposition of treatments effects for stomatal guard cell length of five parents and their hybrid haploid and diploid combinations of maize (Zea mays L.)

Source of variation	df	MS	F
Treatments	16	20680.61	238.21**
Diploid parents	4	2598.61	29.93**
Inducers	1	543.09	6.26*
Supersweet populations	2	1025.33	11.81**
Inducers x Supersweet populations	1	7800.66	89.85**
Diploid F1s	5	224.12	2.58*
Inducers	1	233.31	2.69
Supersweet populations	2	303.98	3.50*
Inducers x Supersweet populations	2	139.67	1.61
Haploid F1s	5	591.11	6.81**
Inducers	1	507.67	5.85*
Supersweet populations	2	1128.44	13.00**
Inducers x Supersweet populations	2	95.51	1.10
(Parents and Diploid F1s) vs. (Haploid F1s)	1	315530.42	3634.42**
Parents vs. Diploid F1s	1	888.65	10.24**
Error	616	86.82	12.27**
Within plant	3165	7.08	-

*, ** Significant at the 5% and 1% levels, respectively.

Table 2. Total number of seedlings and guard cells evaluated, mean stomatal guard cell length (SGCL, in µm), ratio of haploid F1s' to diploid F1s' length (%HD), standard deviation (SD), and confidence interval of treatment means for 95% probability (CI) of the 17 treatments of corn

Treatment	Number		SGCL	(%HD)	SD	CI	
	Seedling	Guard cells				Min	Max
PI4001 (I1)	32	192	52.68abc	-	6.603	50.31	55.05
PI4003 (I3)	32	192	55.06a	-	6.507	52.72	57.39
SD3004 (P4)	32	192	48.02cde	-	3.498	46.76	49.27
SD3005 (P5)	32	192	50.38bcd	-	3.851	49.00	51.76
SD3006 (P6)	32	192	45.76de	-	3.854	44.37	47.14
Diploid F1s - P4×I1	32	192	50.05bcd	-	3.553	48.78	51.33
Diploid F1s - P5×I1	32	192	50.68bcd	-	3.095	49.56	51.79
Diploid F1s - P6×I1	32	192	47.85cde	-	4.068	46.39	49.31
Diploid F1s - P4×I3	32	192	48.86cde	-	3.989	47.43	50.29
Diploid F1s - P5×I3	32	192	48.74cde	-	4.145	47.26	50.23
Diploid F1s - P6×I3	32	192	48.27cde	-	4.011	46.83	49.71
Haploid F1s - P4×I1	28	168	31.46fg	62.9%	2.304	30.57	32.36
Haploid F1s - P5×I1	34	204	34.11f	67.3%	2.700	33.17	35.05
Haploid F1s - P6×I1	20	120	29.89g	62.5%	2.007	28.96	30.83
Haploid F1s - P4×I3	55	330	30.82g	63.1%	2.795	30.07	31.58
Haploid F1s - P5×I3	80	480	31.97fg	65.6%	3.047	31.29	32.65
Haploid F1s - P6×I3	64	384	29.84g	61.8%	3.107	29.07	30.62

Means followed by the same letter in the column do not differ by Tukey's test at 5% probability level.

the seedling leaf sample would be sufficient for the evaluation of each seedling at the V2 stage, having an overall mean standard deviation of 2.66 µm.

Analysis of variance of SGCL data revealed significant effects of treatments and their decomposition (Table 1). The decomposition of treatments referring to the effects of diploid parents showed significant effects for inducers, supersweet populations, and the comparative contrast of these parents. These results, together with the test of means (Table 2), demonstrate that the diploid inducing parents and the supersweet populations differ in terms of SGCL, with the former having a 5.8 µm greater length than the supersweet populations.

There were significant differences between diploid F_1's and between haploid F_1's for SGCL. Significant effects occurred within these decompositions for supersweet parents, at both ploidy levels, and for inducing parents only between haploid F_1's (Table 1). However, there was no significant effect of the inducers x supersweet parents' interaction at either ploidy level. This shows that each parent contributes differently, but uniformly, to reducing or increasing SGCL in the F_1 combinations in which they participate, without specific and differentiated combinations between the parents.

Considering the magnitude of the mean squares and the F test of analysis of variance, the contrast between the mean of the parents and diploid F_1's vs. haploid F_1's was what most contributed to the variation (Table 1). On average, the parents and diploid F_1's showed a SGCL of 49.67 µm, whereas the haploid F_1's averaged 31.32 µm, which is 18.35 µm lower than the overall mean of the evaluated diploids, a considerable reduction of 36.9% (Figure 3). The test of means also set the haploid F_1's apart from diploid genotypes, showing distinct patterns of length between ploidy levels (Table 2), as shown by Choe et al (2012), who also were able to differentiate haploid and diploid individuals using SGCL.

The SGCL of the haploid F_1's of the different supersweet populations exhibited about 61.8% to 67.3% of the length of their respective diploid F_1's (Table 2). Similarly, in the studies by Sekiya et al. (2020), there was also a significant effect of populations and ploidy level of plants on SGCL, but there was no interaction effect between populations and ploidy level, with the haploids showing a SGCL of 52% to 70% compared the diploids for each population. This means that individuals with a SGCL 30% lower than that of diploid F_1's can be classified as haploid, with a safety margin. Molenaar et al. (2019) also observed a significant 27% reduction in the SGCL of haploid seedlings compared with non-haploid plants.

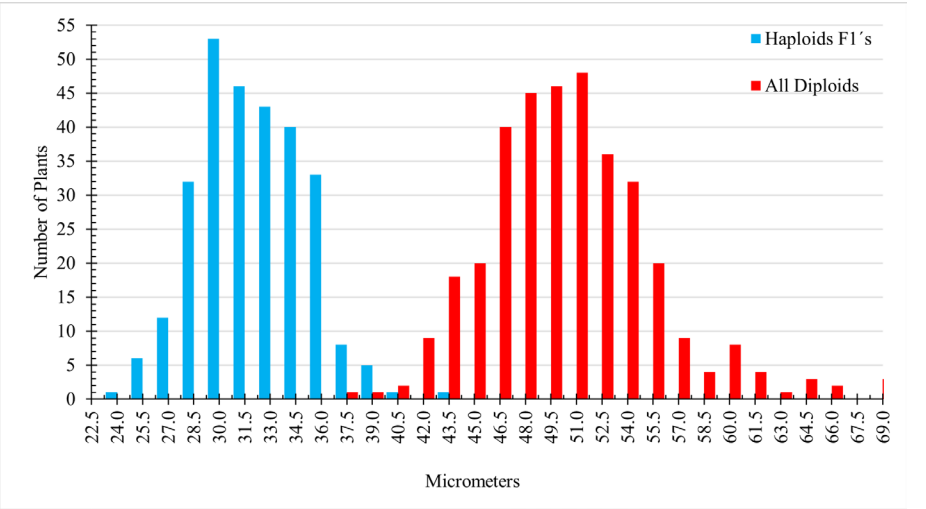

Figure 3. General frequency distribution of mean stomatal guard cell lengths of all evaluated seedlings, with separation by ploidy level only.

Based on the estimates of the confidence intervals of the means (Table 2) and the study of the frequency distribution of the SGCL means of the individual plants within the set of all diploids (Figure 1), which include all parents and diploid F_1s, separated from the set of haploid F_1s, it was possible to define the limit of 37.5 µm for the selection of individuals as haploids. Using this limit, it was found that only one individual out of 352 diploids (0.28%) and 274 individuals out of 281 haploids (97.5%) had a SGCL shorter than 37.5 µm (Figure 3). This shows that the definition of a length limit for SGCL makes it possible to safely accelerate the process of identifying haploids, which is even more useful for populations that inhibit the expression of marker genes such as *R1-nj*. Molenaar et al. (2019) also observed the occurrence of a tenuous marginal overlap in the distribution of SGCL of haploid and non-haploid plants.

The comparison of the means of the parents and diploid F_1 generations (Table 2) revealed a predominance of additive gene action on SGCL, indicating that the mean SGCL of the diploid F_1 generation does not differ significantly from the mean of their parents. Therefore, it is sufficient to evaluate the diploid and haploid F_1 generations to construct SGCL differentiation limits for the early selection of haploid seedlings.

In association with observations of seedling vigor and morphology, the measurement of a representative guard cell of a leaf sample and its comparison as a threshold value of SGCL effectively help to differentiate haploid and diploid seedlings in a simple, fast, non-destructive, and low-cost manner. Thus, mainly for chromosome duplication methods in seedlings at stage V2 (Eder and Chalyk 2002, Chaikan et al. 2020), early selection using SGCL allows the elimination of diploids before treatment with antimitotic agents. This can increase the efficiency of production of double-haploids in maize, being an alternative to obtain DH's in populations with genes that inhibit the expression of the *R1-nj* gene in the seed.

CONCLUSIONS

Haploid inducers and supersweet corn genotypes and their hybrid combinations, between groups, have different guard cell lengths.

The evaluation of guard cell length in leaf samples of V2 supersweet corn seedlings makes it possible to efficiently differentiate haploid from diploid individuals.

There is no interaction between haploid inducers and supersweet populations that results in a differentiated contribution to the increase or decrease in SGCL in the F_1 generations.

It is sufficient to evaluate the diploid and haploid F_1 generations to construct SGCL differentiation limits for the early selection of haploid seedlings.

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