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**Development of haploid Inducers and characterization of unilateral cross-
incompatibility in popcorn germplasm**

Jean Paulo Aparecido da Silva
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Thesis submitted to the Genetics and Breeding Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Jose Marcelo Soriano Viana

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ABSTRACT

SILVA, Jean Paulo Aparecido da, D.Sc., Universidade Federal de Viçosa, August, 2026. **Development of haploid inducers and characterization of unilateral cross-incompatibility in popcorn germplasm.** Adviser: Jose Marcelo Soriano Viana.

Although unilateral cross-incompatibility (UCI) represents a significant barrier to the implementation of in vivo induction of maternal haploids in popcorn, the main limitation remains the absence of efficient haploid inducers adapted to this germplasm. This study aimed to evaluate the genetic potential of the Krasnodar Haploid Inducer (KHI) population to increase the rate of haploid induction (HIR) and to characterize the UCI between KHI and popcorn germplasm. HIR was evaluated in two cycles of mass selection of KHI, using tester hybrids, and in S progenies derived from selected plants from both cycles (CI and CII). The UCI (Unit of Competence) was evaluated through direct (KHI $\times$ popcorn) and reciprocal (popcorn $\times$ KHI) controlled crosses, involving 56 tropical and temperate popcorn lines. The experiment was conducted in a completely randomized design with three replications. The HIR (Human Influence Ratio) was analyzed using a generalized linear mixed model, while the compatibility probabilities of the crosses were estimated by generalized linear models with a logit link function. Compatibility was defined as the probability of occurrence of compatible crosses, and the differences between direct and reciprocal crosses were used to characterize the UCI. The posterior mean of the HIR was 0.28%, 0.30%, and 0.34% in Cycle I, Cycle II, and in the S progenies, respectively. Additive genetic variance ranged from 2.75 to 4.39, and heritability ranged from 0.39 to 0.51. The highest predicted HIR was 5.97% in Cycle II. The compatibility probabilities in self-fertilizations were 89.6% and 93.6% for tropical and temperate germplasm, respectively. The compatibility probabilities of crosses were 53.7% and 82.8% for direct and reciprocal crosses, respectively, with greater asymmetry in temperate germplasm (45.4 percentage points) than in tropical germplasm (12.6 percentage points). The observed frequencies of compatibility varied widely among the lines, from 0.0% to 95.2% in direct crosses and from 25.0% to 100.0% in reciprocal crosses. Compatibility in reciprocal crosses was generally higher than that observed in direct crosses, particularly among temperate lines, although some tropical lines showed the opposite pattern. The low average HIR limits the use of KHI alone for the development of efficient haploid inducers. Crossing KHI with other sources of haploid inducers or with adapted non-inducing germplasm constitutes a strategy to generate

transgressive segregants and enable the selection of haploid inducers with higher HIR. The compatibility patterns observed between the different crossing directions highlight the occurrence of UCI and reveal variation between germplasm groups, populations, and lines. These results are relevant for identifying the incompatibility systems present in popcorn germplasm, particularly for determining which system is associated with the incompatibility observed in tropical germplasm and confirming the presence of the system. *Ga1* in temperate germplasm, whose origin suggests the occurrence of this system. The diversity of compatibility patterns observed may also support strategies to overcome crossing barriers between the haploid inducer and popcorn.

Keywords: *Zea mays* L. var. Evert; maternal haploid; gametophytic factors;

RESUMO

SILVA, Jean Paulo Aparecido da, D.Sc., Universidade Federal de Viçosa, agosto de 2026. **Desenvolvimento de indutores de haploides e caracterização da incompatibilidade cruzada unilateral em germoplasma de milho-pipoca.** Orientador: Jose Marcelo Soriano Viana.

Embora a incompatibilidade cruzada unilateral (UCI) represente uma importante barreira para a implementação da indução *in vivo* de haploides maternos em milho-pipoca, a principal limitação ainda é a ausência de indutores de haploides eficientes e adaptados a esse germoplasma. Neste estudo, objetivou-se avaliar o potencial genético da população Krasnodar Haploid Inducer (KHI) para o aumento da taxa de indução de haploides (HIR) e caracterizar a UCI entre a KHI e o germoplasma de milho-pipoca. A HIR foi avaliada em dois ciclos de seleção massal da KHI, utilizando híbridos testadores, e em progênies S derivadas de plantas selecionadas de ambos os ciclos (CI e CII). A UCI foi avaliada por meio de cruzamentos controlados diretos (KHI $\times$ milho-pipoca) e recíprocos (milho-pipoca $\times$ KHI), envolvendo 56 linhagens de milho-pipoca tropicais e temperadas. O experimento foi conduzido em delineamento inteiramente casualizado, com três repetições. A HIR foi analisada por meio de um modelo linear generalizado misto, enquanto as probabilidades de compatibilidade dos cruzamentos foram estimadas por modelos lineares generalizados com função de ligação logit. A compatibilidade foi definida como a probabilidade de ocorrência de cruzamentos compatíveis, e as diferenças entre os cruzamentos diretos e recíprocos foram utilizadas para caracterizar a UCI. A média posterior da HIR foi de 0,28%, 0,30% e 0,34% no Ciclo I, Ciclo II e nas progênies S, respectivamente. A variância genética aditiva variou de 2,75 a 4,39, e a herdabilidade variou de 0,39 a 0,51. A maior HIR predita foi de 5,97% no Ciclo II. As probabilidades de compatibilidade em autofecundações foram de 89,6% e 93,6% para os germoplasmas tropical e temperado, respectivamente. As probabilidades de compatibilidade dos cruzamentos foram de 53,7% e 82,8% para os cruzamentos diretos e recíprocos, respectivamente, com maior assimetria no germoplasma temperado (45,4 pontos percentuais) do que no germoplasma tropical (12,6 pontos percentuais). As frequências observadas de compatibilidade variaram amplamente entre as linhagens, de 0,0% a 95,2% nos cruzamentos diretos e de 25,0% a 100,0% nos cruzamentos recíprocos. A compatibilidade nos cruzamentos recíprocos foi geralmente superior à observada nos cruzamentos diretos, particularmente entre as linhagens temperadas, embora algumas linhagens tropicais tenham apresentado o padrão oposto.

média limita o uso do KHI isoladamente para o desenvolvimento de indutores de haploides eficientes. O cruzamento do KHI com outras fontes de indutores de haploides ou com germoplasma não indutor adaptado constitui uma estratégia para gerar segregantes transgressivos e possibilitar a seleção de indutores de haploides com maior HIR. Os padrões de compatibilidade observados entre as diferentes direções de cruzamento evidenciam a ocorrência de UCI e revelam variação entre grupos de germoplasma, populações e linhagens. Esses resultados são relevantes para identificar os sistemas de incompatibilidade presentes no germoplasma de milho-pipoca, particularmente para determinar qual sistema está associado à incompatibilidade observada no germoplasma tropical e confirmar a presença do sistema *Ga1* no germoplasma temperado, cuja origem sugere a ocorrência desse sistema. A diversidade de padrões de compatibilidade observada também pode subsidiar estratégias para superar as barreiras de cruzamento entre o indutor de haploides e o milho-pipoca.

Palavras-chave: *Zea mays* L. var. *Evert*; haploide materno; fatores gametofíticos;

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1 INTRODUCTION

Efficient haploid production is a key factor for the successful implementation of doubled haploid (DH) technology in breeding programs (Chaikam *et al.*, 2018; Khammona *et al.*, 2024; Prigge *et al.*, 2012a). In maize, haploid production is primarily achieved through *in vivo* maternal haploid induction, in which specialized inducer lines are used as pollen donors to generate maternal haploids (Chaikam *et al.*, 2019). Early efforts to increase maternal haploid induction rates (HIR) led to the identification of the pioneering inducer line Stock 6 (Coe, 1959). Building on this genetic background, breeding programs worldwide have developed maternal haploid inducers with higher HIR and improved agronomic adaptation (Röber; Gordillo; Geiger, 2005; Rotarenco *et al.*, 2010).

In parallel with the development of new haploid inducers, several studies have investigated the genetic basis of HIR, leading to the identification of quantitative trait loci (QTLs) associated with this trait. Deimling, Röber and Geiger (1997) identified two QTLs on chromosomes 1 and 2, which together explained 17.9% of the phenotypic variation. Subsequent studies confirmed chromosome 1 as the major genomic region controlling HIR (Barret; Brinkmann; Beckert, 2008). Further mapping analyses conducted by Prigge *et al.* (2012b) refined this major locus, designated *qhir1*, and identified additional modifier QTLs distributed across chromosomes 3, 4, 5, 7, and 9, including *qhir8*, which contributed substantially to HIR variation. Later, Dong *et al.* (2013), followed by Hu *et al.* (2016) and Nair *et al.* (2017), further refined the *qhir1* region, providing the foundation for the subsequent identification of the causal gene underlying this locus.

Advances in fine mapping, molecular characterization, and functional validation have enabled the identification of genes underlying major QTLs associated with HIR. The characterization of *ZmMTL*, underlying the *qhir1* locus, and *ZmDMP*, underlying the *qhir8* locus, represented a major advance in understanding the molecular basis of haploid induction in maize (Gilles *et al.*, 2017; Kelliher *et al.*, 2017; Liu *et al.*, 2017). *ZmMTL* encodes a patatin-like phospholipase, and loss-of-function mutations in this gene are associated with higher HIR. Furthermore, Jiang *et al.* (2022) demonstrated that loss of *ZmMTL* function disrupts sperm cell redox homeostasis, resulting in increased reactive oxygen species accumulation, paternal DNA damage, and subsequent paternal genome elimination after fertilization, ultimately leading to haploid embryo formation.

Using a diversity panel of 159 haploid inducer lines, Trentin *et al.* (2023) identified genomic regions associated with HIR across six of the ten maize chromosomes, further supporting the polygenic architecture of this trait. Gain *et al.* (2025) evaluated advanced haploid

inducer lines up to the F₅ generation carrying different allelic combinations of *ZmMTL* and *ZmDMP* (*mtlmtl/DMPDMP*, *MTLMTL/dmpdmp*, and *mtlmtl/dmpdmp*) and demonstrated a synergistic interaction between the *mtl* and *dmp* mutant alleles. Double-mutant lines (*mtlmtl/dmpdmp*) exhibited a mean HIR of 8.88%, whereas lines carrying only the *mtl* mutation (*mtlmtl/DMPDMP*) averaged 3.61%. Similarly, Khammona *et al.* (2025) demonstrated the effectiveness of marker-assisted selection (MAS) targeting the major-effect loci *qhir1/ZmMTL* and *qhir8/ZmDMP* for developing haploid inducer lines. Simultaneous selection for these loci in segregating populations enabled the identification of *qhir1*⁺/*qhir8*⁺ genotypes with HIR values one to three times higher than those carrying *qhir1*⁺ alone. Collectively, these studies demonstrate that, although *ZmMTL* and *ZmDMP* are major determinants of HIR, the trait remains strongly influenced by the underlying genetic background.

The successful application of maternal haploid induction depends on the ability of the inducer to fertilize the female germplasm. In popcorn, however, reproductive barriers can prevent fertilization between plants carrying incompatible allelic combinations, thereby resulting in unilateral cross-incompatibility (UCI). Three major UCI loci have been described in *Zea mays*, namely gametophyte factor 1 (*Gal*), gametophyte factor 2 (*Ga2*), and teosinte crossing barrier 1 (*Tcb1*) (Evans; Kermicle, 2001; Kermicle, 2006; Kermicle; Evans, 2010).

Among these loci, *Gal* is particularly relevant to popcorn because of its widespread occurrence in popcorn germplasm and its use in commercial production systems to maintain genetic purity (Hallauer, 2000). At each UCI locus, the -S haplotypes (e.g., *Gal-S*, *Ga2-S*, and *Tcb1-S*) carry both male and female functions, whereas the corresponding -M haplotypes carry only the male function. In contrast, the recessive haplotypes (*gal*, *ga2*, and *tcb1*) lack both male and female functions. Consequently, successful fertilization depends on the combination of alleles carried by the female parent and the pollen donor. For example, silks carrying *Gal-S* prevent fertilization by *gal* pollen, whereas *Gal-S* pollen can successfully fertilize *gal* silks, making the cross compatible only in this direction (Chen *et al.*, 2022; Cryan *et al.*, 2025; Lu; Kermicle; Evans, 2014; Wang *et al.*, 2022). At the molecular level, the male and female functions are encoded by distinct pollen- and silk-expressed pectin methylesterases that regulate pollen tube growth (Bapat *et al.*, 2023; Bapat; Scott, 2024, 2024; Moran Lauter; Holland; Scott, 2025; Zhang *et al.*, 2018, 2023).

However, although several haploid inducer lines have been described in the literature and are available in germplasm collections, access to elite inducers used in commercial breeding programs remains limited and is often restricted by licensing agreements and germplasm transfer policies. Furthermore, because UCI can prevent successful fertilization of popcorn

germplasm by haploid inducer pollen, understanding its occurrence is essential for the effective deployment and utilization of haploid inducer.

2 OBJECTIVES

The objectives of this study were to evaluate the genetic potential of Krasnodar Haploid Inducer (KHI) for HIR improvement and to characterize UCI between the KHI population and popcorn germplasm.

3 MATERIAL AND METHODS

3.1 Haploid induction rate evaluation in the KHI population

3.1.1 Field trials

This study was conducted at an experimental area at the Federal University of Viçosa, Minas Gerais, Brazil. Selection in the KHI inducer population was based on HIR estimates, except for Cycle 0, in which selection was based on the mean observed HIR across the two testers. Throughout the selection cycles, plants were grown in an isolated field under open pollination, and ears were harvested individually to generate half-sib progenies. In the 2021/2022 growing season, Cycle 0 comprised the evaluation of 1600 individuals using the hybrids 2B710PW and MG580PW as testers. The 389 best plants were selected based on their mean observed HIR across the two testers and were used to generate the half-sib progenies evaluated in Cycle I.

In Cycle I, conducted in the 2022/2023 growing season, 2821 individuals were evaluated, with progeny sizes ranging from 1 to 15 plants. Based on HIR estimates, the 286 best plants were selected to generate Cycle II. In Cycle II, conducted in the 2023/2024 growing season, 1035 individuals from 286 progenies were evaluated, with progeny sizes ranging from 1 to 13 plants. In the 2024/2025 growing season, 232 S0 progenies derived from plants selected in Cycles I and II were evaluated, totaling 1,171 individuals, with 1 to 15 plants per progeny. At this stage, plants were evaluated for HIR and subsequently self-pollinated to obtain S1 progenies.

The HIR was evaluated through crosses between individuals from the KHI inducer population, used as pollen donors, and single-cross hybrids, used as female parents. KHI is a public synthetic population developed as a haploid inducer, also known as KEMS (Krasnodarskiy Embryo Marker Synthetic) (Shatskaya et al., 1994). To synchronize flowering, the tester hybrids were sown at two planting dates: the first planting was performed on day 0, and the second planting was performed seven days later, on day 7. The KHI inducer progenies

were sown on day 15. This staggered planting schedule was adopted to promote synchronization of flowering between the tester hybrids and the inducer progenies and to ensure adequate pollen availability for the controlled crosses.

The tester hybrids were planted in 4-m rows, with a tester-to-inducer progeny row ratio of approximately 2:1. Both tester and inducer progenies were established at the same planting density of five plants per meter. Pollen from each inducer individual was used for manual pollination of the tester ears. The tester plants were detasseled to ensure that the ears used for HIR evaluation received pollen exclusively from the inducer population and to prevent contamination of plants maintained under open pollination. The resulting seeds were visually classified, and putative haploid seeds were identified by the presence of purple endosperm and an unpigmented embryo. The HIR was estimated for each individual as the proportion of putative haploid seeds relative to the total number of seeds evaluated (Nanda; Chase, 1966).

3.1.2 Statistical analysis

We fitted a generalized linear mixed model with a binomial distribution and a logit link function to analyze HIR. The haploid induction rate was modeled as $y \sim \text{Binomial}(n, \rho)$, where y is the number of haploid seeds, n is the total number of seeds evaluated, and ρ is the probability of haploid induction. The logit link function was defined as $\text{logit}(\rho) = \ln[\rho/(1 - \rho)]$, relating the probability of success to the linear predictor. The model was specified as $\text{logit}(\rho) = X\beta + Zu$, where β is the vector of fixed effects of selection cycles and u is the vector of additive genetic effects of individuals within selection cycles. The matrices X and Z are the corresponding incidence matrices for fixed and random effects, respectively. The additive genetic effects were assumed to follow a multivariate normal distribution, $u \sim N(0, \Sigma_g \otimes A)$, where Σ_g is a diagonal matrix containing the additive genetic variances for each selection cycle and A is the additive genetic relationship matrix computed using the AGHmatrix package (Amadeu *et al.*, 2023).

The additive genetic variance components were estimated using Markov chain Monte Carlo (MCMC), with 1000000 iterations, a burn-in period of 10000 iterations, and a thinning interval of 100 iterations, resulting in 9900 posterior samples for parameter inference. For the logit link function, the residual variance was fixed at the theoretical value of $\pi^2/3$. Predicted additive values were obtained from the posterior means. Heritability estimates were calculated as the ratio between the additive variance and the residual variance. Statistical analyses were performed using the MCMCglmm package (Hadfield, 2010). Convergence was assessed by visual inspection of trace plots and Geweke's test conducted using the coda package in R.

3.2 Unilateral cross-incompatibility evaluation

3.2.1 Plant materials

We evaluated 56 popcorn inbred lines, including eight from each of two tropical populations (Viçosa C4 and Beija-Flor C4) and eight from each of five temperate populations (UFV-MP1 to UFV-MP5). The haploid inducer population KHI, presumed to carry the *ga1/ga1* allele, was used as the tester. Viçosa C4 and Beija-Flor C4 were developed after four cycles of half-sib selection from their respective base populations. The temperate populations were derived by recombining the single crosses P622, P625, P802, AP2501, and AP4502, developed by the Agricultural Alumni Seed Improvement Association (Romney, IN, USA).

3.2.2 Controlled crosses and compatibility assessment

The experiment was conducted during the 2022/2023 growing season in a completely randomized design with three replicates. Each plot consisted of a 3-m row with a final stand of 15 plants. The popcorn inbred lines were sown in a single planting window, whereas the KHI was sown on two dates to ensure floral synchrony and pollen availability. The first KHI sowing was performed simultaneously with the popcorn inbred lines, and the second was performed when plants from the first sowing reached the seedling emergence stage.

We performed controlled crosses between the popcorn inbred lines and the KHI, including direct crosses (KHI ♂ × popcorn ♀) and reciprocal crosses (popcorn ♂ × KHI ♀). We also self-pollinated at least one plant per replicate to assess self-compatibility. Ears were classified as self-compatible or reciprocal-cross compatible when full seeds were produced. Self-compatibility and reciprocal-cross compatibility probabilities were estimated as the probabilities of compatible ears among all self-pollinated and reciprocal-crossed ears evaluated, respectively.

For direct crosses, compatibility was assessed based on the expression of the *R1-nj* marker. An ear was classified as compatible when at least one seed expressed the marker, indicating the contribution of KHI pollen. Direct-cross compatibility probability was estimated as the probability of compatible ears among all direct-crossed ears evaluated. These binary outcomes were used to estimate compatibility probabilities. Differences between direct- and reciprocal-cross compatibility were used to assess cross-direction asymmetry, with significant asymmetry considered evidence of UCI.

3.2.3 Statistical analysis

We fitted generalized linear models with a binomial distribution and a logit link function to analyze cross-compatibility between the popcorn inbred lines and KHI. The analyses were performed at three hierarchical levels of genetic grouping: germplasm origin, population, and inbred line. Only direct and reciprocal crosses were included in these analyses. The response variable was modeled as $y \sim \text{Binomial}(n, \rho)$, where y is the number of compatible ears, n the number of ears evaluated, and ρ is the probability of cross-compatibility. The logit link function was defined as $\text{logit}(\rho) = \ln[\rho/(1 - \rho)]$. The general model structure was expressed as: $\text{logit}(\rho_{ijk}) = \beta_0 + R_i + G_j + C_k + (G \times C)_{jk}$ where ρ_{ijk} is the probability of a compatible cross; β_0 is the intercept; R_i is the fixed effect of replicate; G_j represents the effect of the genetic grouping (germplasm origin, inbred line, or population); C_k represents the cross direction; and $(G \times C)_{jk}$ represents the interaction between genetic grouping and cross direction. Model goodness of fit was assessed by visual inspection of half-normal plots with a simulated envelope using the *hnp* package (Demétrio; Hinde; Moral, 2014; Moral; Hinde; Demétrio, 2017). The significance of model effects was assessed using likelihood ratio tests based on the chi-square distribution. Estimated probabilities of compatibility and 95% confidence intervals were obtained using estimated marginal means implemented in the *emmeans* package (Lenth; Piaskowski, 2026). All statistical analyses were performed in R, using a significance level of $\alpha = 0.05$.

4 RESULTS

4.1 Haploid induction rate improvement

The MCMC chains showed satisfactory convergence across selection cycles. Visual inspection of the trace plots indicated stable and well-mixed chains, while the estimated marginal posterior densities showed consistent distributions without evident irregularities. These results were further supported by Geweke's diagnostic test, which indicated no evidence of lack of convergence. The trace plots and estimated marginal posterior densities for the additive genetic variance components are presented in Figure 1.

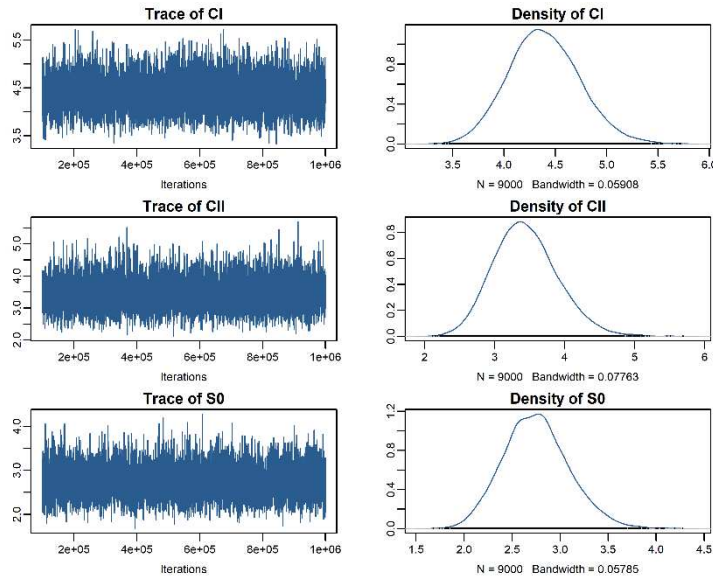


Figure 1 Trace plots (left) and estimated marginal posterior densities (right) for the additive genetic variance components across selection cycles.

In Cycle 0, we observed an average HIR of 1.0% among the 389 best-performing plants, considering both testers (MG580PW and 2B710PW). Among the 1,600 KHI plants evaluated in Cycle 0, the mean HIR was 0.26% for MG580PW and 0.27% for 2B710PW. The Pearson correlation coefficient between the two testers was approximately 0.69. The posterior mean HIR was 0.28% and 0.30% in Cycles I and II, respectively (Figure 2). The S₀ progenies derived from plants selected in Cycles I and II showed a posterior mean HIR of 0.34%. The highest predicted HIR values among progenies were 4.97% and 5.97% in Cycles I and II, respectively, whereas the highest predicted HIR among S₀ progenies was 4.52%.

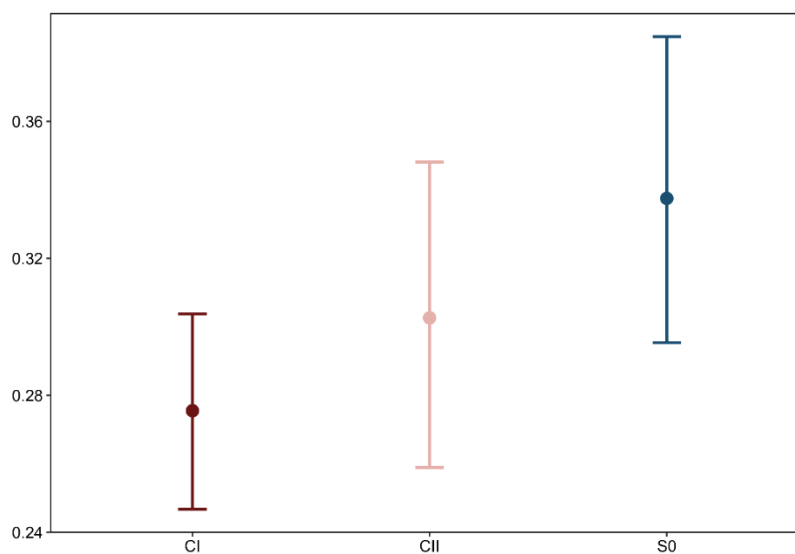


Figure 2 Posterior estimates of haploid inducer rate and with 95% highest posterior density intervals.

Heritability decreased by approximately 10% and 20% in Cycles II and S_0 , respectively, relative to Cycle I (Figure 3). Additive genetic variance followed a similar pattern, decreasing by approximately 20% and 37% in Cycles II and S_0 , respectively. Importantly, we continued to observe a high frequency of individuals with no haploid seeds throughout the selection process. This frequency decreased from 72.2% in Cycle I to 69.3% in Cycle II and 63.8% in S_0 . Despite this reduction, a substantial proportion of individuals still showed no observed haploid induction (data not shown). We evaluated approximately 63.3% fewer individuals in Cycle II than in Cycle I. High temperatures during the 2023/2024 growing season likely contributed to pollen production failure and consequently reduced the number of individuals available for evaluation.

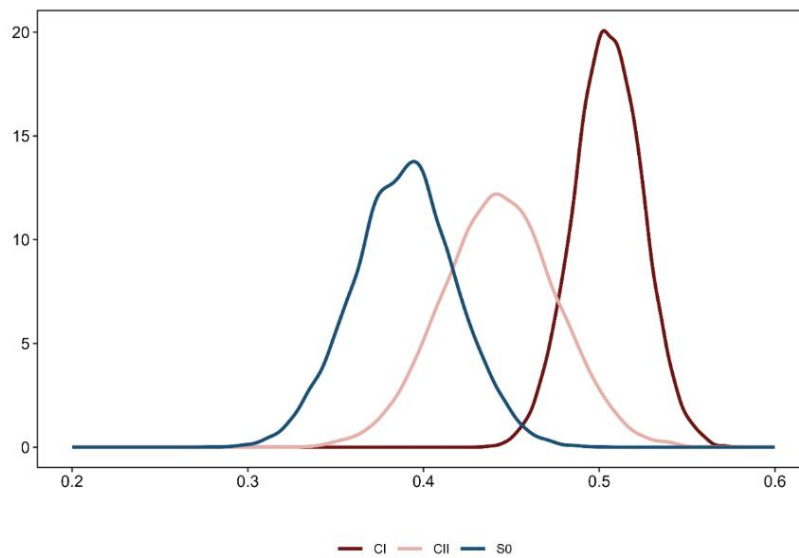


Figure 3 Posterior distributions of heritability for haploid inducer rate.

4.2 Unilateral cross-incompatibility

We evaluated a total of 1,744 direct and reciprocal crosses to quantify cross-compatibility (data not shown). Most evaluated crosses resulted in either complete cross-failure (zero seeds) or complete seed set, whereas 218 crosses produced intermediate numbers of seeds, ranging from 1 to 64 seeds per ear (Figure 4). These intermediate cases made their classification as compatible or incompatible less straightforward. The threshold for classifying crosses as compatible varies among studies, with some adopting a minimum of three seeds per ear (Moran Lauter; Holland; Scott, 2025) and others five seeds per ear (Zhang *et al.*, 2018), although the rationale for these thresholds was not clearly stated. Therefore, we adopted a minimum threshold of one seed per ear (≥ 1 seed) to classify crosses as compatible.



Figure 4 Representative popcorn ears from crosses with KHI illustrating different compatibility patterns, shown from left to right as complete compatibility, with all kernels expressing the *R1- η j* marker and indicating KHI pollen contribution; complete incompatibility, with no kernels produced; and intermediate compatibility, with only a subset of kernels expressing the *R1- η j* marker.

Self-compatibility rates were high across germplasm and populations. Mean self-compatibility was 89.1% in tropical germplasm, ranging from 84.0% in Beija-Flor C4 to 94.1% in Viçosa C4, and 93.5% in temperate germplasm, ranging from 86.2% in UFV-MP5 to 97.1% in UFV-MP1 (Figure 5). Because we performed self-pollinations alongside the crosses, these results provide an indirect indication of adequate reproductive performance and support the effectiveness of the pollination procedures we used in the experiment. Although self-compatibility does not directly measure pollen viability, the high success rates of self-pollination provide a useful reference for interpreting the compatibility results of the KHI $\times$ popcorn crosses.

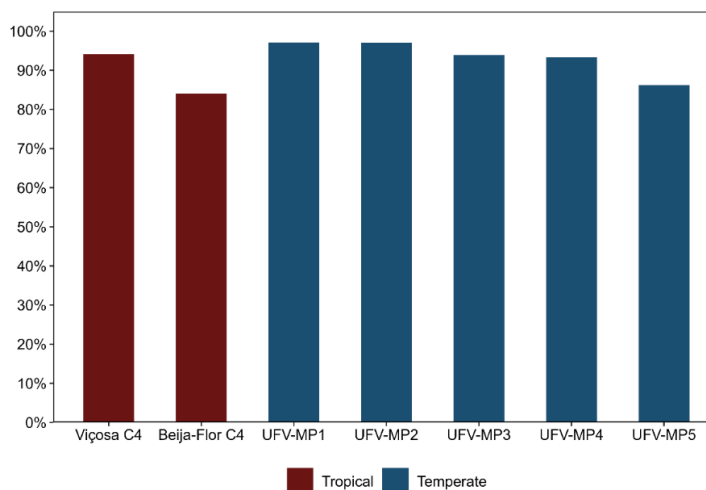


Figure 5 Average self-compatibility across popcorn populations.

The binomial GLMs showed an adequate fit across the three levels of analysis (Supplementary Figure 1). The estimated cross-compatibility probabilities were 53.7% and 82.8% for direct and reciprocal crosses, respectively (Figure 6). We found significant effects of germplasm origin, cross direction, and their interaction ($P < 0.001$; Supplementary Table 1).

The magnitude of cross-direction asymmetry was 45.4 percentage points in temperate germplasm, compared with 12.6 percentage points in tropical germplasm.

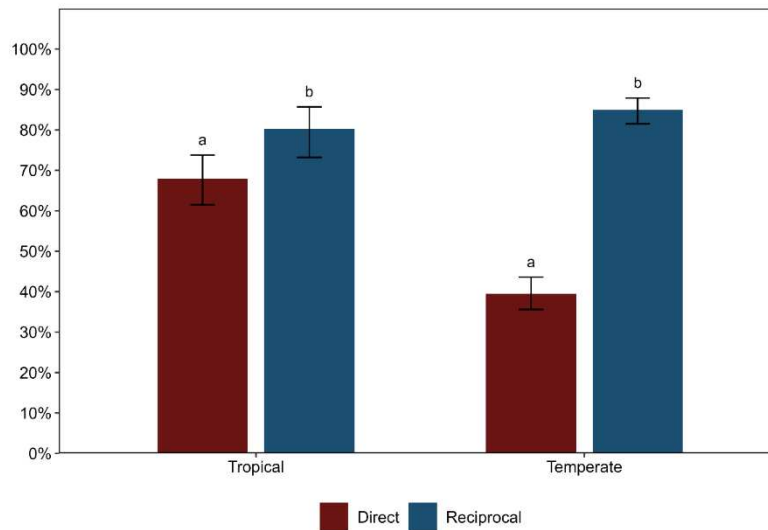


Figure 6 Predicted probabilities of compatibility for direct (KHI ♂ × popcorn ♀) and reciprocal (popcorn ♂ × KHI ♀) crosses within each germplasm origin. Bars represent predicted probabilities with 95% confidence intervals. Different letters indicate significant differences between cross directions according to test ($\alpha = 0.05$).

We found significant differences between cross directions in six of the seven populations: Beija-Flor C4, UFV-MP1, UFV-MP2, UFV-MP3, UFV-MP4, and UFV-MP5 (Figure 7). Direct-cross compatibility ranged from 30.8% (UFV-MP1) to 68.3% (Viçosa C4), whereas reciprocal-cross compatibility ranged from 77.8% (UFV-MP5) to 92.5% (UFV-MP2). The largest cross-direction asymmetries occurred in UFV-MP3 (55.0 percentage points), UFV-MP1 (52.4 percentage points), and UFV-MP4 (49.6 percentage points). Viçosa C4 was the exception, showing no significant difference between direct (68.3%) and reciprocal (78.4%) crosses. Overall, cross-direction asymmetry was greater in temperate populations.

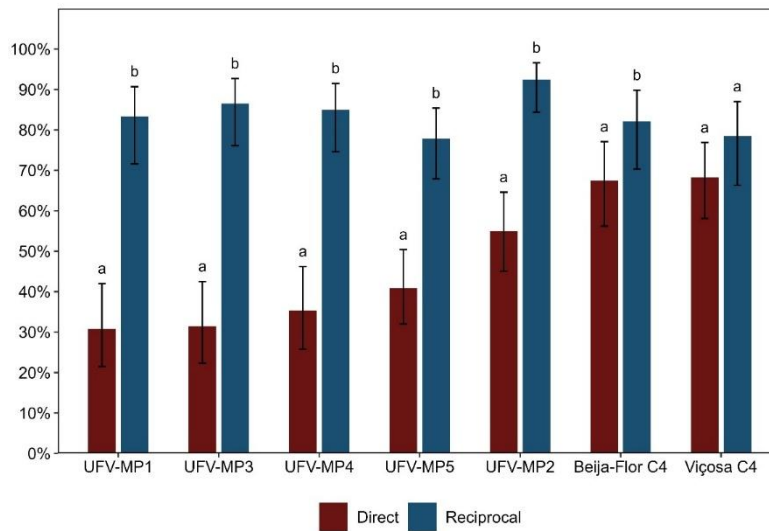


Figure 7 Predicted probabilities of compatibility for direct (KHI ♂ × popcorn ♀) and reciprocal (popcorn ♂ × KHI ♀) crosses within each population. Bars represent predicted probabilities with 95% confidence intervals. Different letters indicate significant differences between cross directions according to Sidak's test ($\alpha = 0.05$).

Compatibility varied significantly among inbred lines ($P < 0.001$), and the inbred line × cross-direction interaction was also significant ($P < 0.001$), whereas the main effect of cross direction was not significant ($P = 0.558$) (Supplementary Table 1). Observed compatibility frequencies varied among inbred lines, ranging from 0.0% to 95.2% for direct crosses (Figure 9) and from 25.0% to 100.0% for reciprocal crosses (Figure 8). In most inbred lines, particularly among the temperate lines, reciprocal-cross compatibility exceeded direct-cross compatibility. The largest differences occurred in lines 21-1850-3 from UFV-MP1 (0.0% direct vs. 88.9% reciprocal), 21-479-6 from UFV-MP3 (11.1% vs. 100.0%), 21-1727-4 from UFV-MP1 (11.1% vs. 90.0%), 21-307-3 from UFV-MP5 (5.6% vs. 87.5%), and 21-1255-1 from UFV-MP2 (17.6% vs. 90.9%). In contrast, some tropical lines showed the opposite pattern, with higher compatibility in direct crosses: 15-1149-4 from Viçosa C4 (92.9% vs. 50.0%), 15-1155-9 from Beija-Flor C4 (92.3% vs. 58.3%), 15-1092-7 from Viçosa C4 (83.3% vs. 60.0%), and 15-1073-2 from Viçosa C4 (95.2% vs. 75.0%).

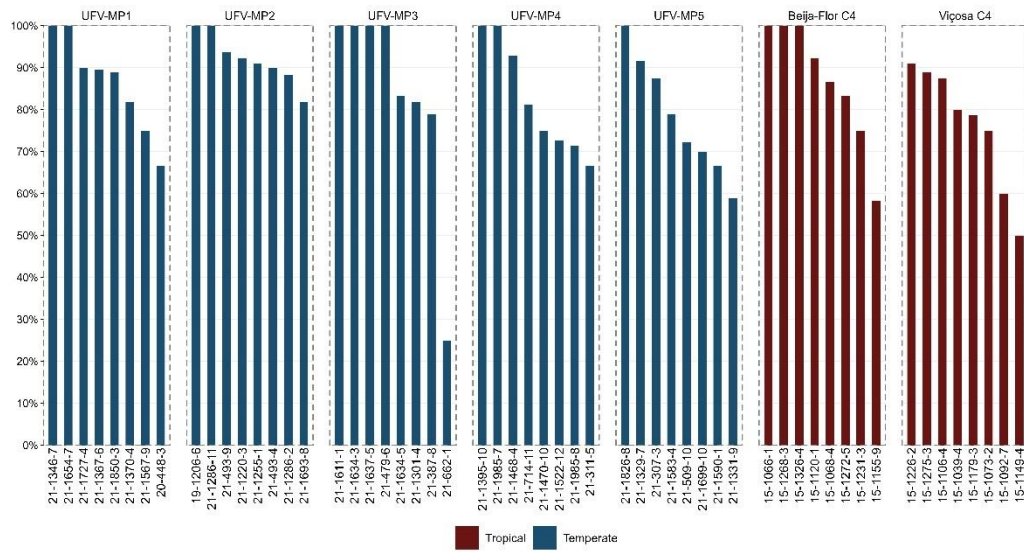


Figure 8 Frequency of compatibility (%) for reciprocal crosses (popcorn ♀ × KHI ♂) among inbred lines evaluated within each germplasm class and population.

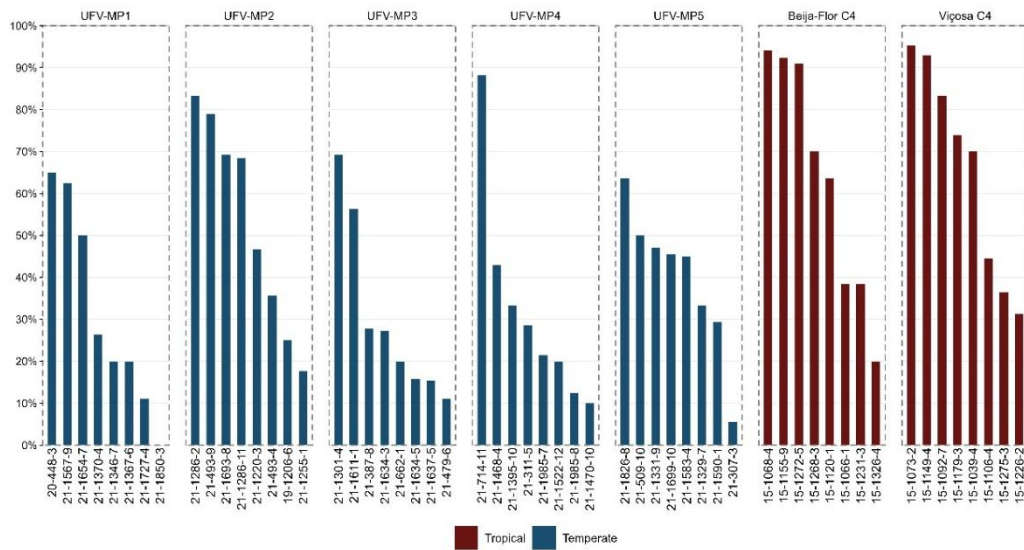


Figure 9 Frequency of compatibility (%) for direct crosses (KHI ♂ × popcorn ♀) among inbred lines evaluated within each germplasm and population.

5 DISCUSSION

Maternal *in vivo* haploid induction provides an efficient approach for haploid production and the implementation of DH technology in maize breeding programs (Chaikam *et al.*, 2019). However, the success of this technology largely depends on the availability of haploid inducers with high HIR (Chaikam *et al.*, 2018; Prigge *et al.*, 2012a). Several improved haploid inducers have been developed, with HIR values generally exceeding 10%. Couto *et al.* (2015) reported a mean HIR of 8.3% across four single crosses and their respective F₂ generations using KHI as the haploid inducer. In contrast, we observed that the mean HIR of the KHI remained below

1% after two cycles of mass selection, although some individuals exhibited predicted HIR values approaching 6%. The lower HIR observed in the KHI compared with previous reports may be associated with differences in environmental conditions and interactions between the haploid inducer genotype and the female germplasm used as the haploid source (De La Fuente *et al.*, 2018; Ren *et al.*, 2022; Uliana Trentin *et al.*, 2022).

Several studies have reported heritability estimates for HIR; however, direct comparisons among studies should be interpreted with caution. Because heritability is a population-specific parameter, differences in the genetic background of the populations evaluated can influence its magnitude (Hill; Mackay, 2004). In addition, HIR is expressed as a proportion and typically exhibits a non-Gaussian distribution. Consequently, different statistical approaches have been used to estimate genetic parameters for HIR, including analyses on the observed scale (Almeida *et al.*, 2020), data transformation (Dermail *et al.*, 2021), and generalized linear mixed models that account for the underlying distribution of the trait (Trentin *et al.*, 2023b). Here, we estimated heritability using a generalized linear mixed model, obtaining values ranging from 0.39 to 0.51. Similarly, Ribeiro *et al.* (2020) estimated heritability in inbred progenies and reported relatively low estimates (0.09–0.22). In contrast, Prigge *et al.* (2012b) reported higher heritability estimates (0.32–0.80). Despite these methodological differences, the moderate heritability estimates obtained in this study indicate that additive variance contributes to the observed variation in HIR.

The estimated additive genetic variance, as indicated by the 95% HPD intervals excluding zero, confirms the presence of exploitable genetic variation within the KHI, with estimates of 4.39 (3.74–5.08), 3.44 (2.58–4.35), and 2.75 (2.10–3.43) across evaluation cycles. However, the presence of genetic variation alone does not necessarily translate into genetic gains. In successful haploid inducer breeding programs, major improvements in HIR have generally been achieved by broadening the genetic base through crosses among distinct inducer sources or between haploid inducers and elite non-inducer germplasm, rather than relying exclusively on selection within a single population (Chaikam *et al.*, 2018; Chang; Coe, 2009; Prigge *et al.*, 2012a; Röber; Gordillo; Geiger, 2005). The presence of haploid induction-associated alleles at *qhir1/ZmMTL* represents a major determinant of haploid induction ability, whereas allelic variation at *qhir8/ZmDMP* can further enhance HIR when combined with *qhir1* (Chen *et al.*, 2020). Khammona *et al.* (2024) demonstrated that simultaneous selection for *qhir1* and *qhir8* increased HIR by 1- to 3-fold compared with genotypes carrying *qhir1* alone. Beyond these major genes, several studies have identified additional QTLs associated with HIR, including *qhir11* and *qhir12* on chromosome 1 (Hu *et al.*, 2016; Nair *et al.*, 2017; Prigge

et al., 2012b), as well as loci associated with genes such as *ZmDMP/DUF679* (Zhong *et al.*, 2019), *ZmPLD3* (Li *et al.*, 2021), and *ZmPOD65* (Jiang *et al.*, 2022).

Concerning UCI, we observed greater asymmetry in compatibility probabilities between direct and reciprocal crosses in temperate popcorn than in tropical popcorn germplasm, indicating a stronger directional effect of the pollen–pistil barrier in temperate populations. This pattern may reflect the widespread occurrence of the *Gal* system in popcorn, where compatibility barriers contribute to maintaining the genetic purity of commercial popcorn seed (Goodman *et al.*, 2021). However, variation in compatibility responses was also detected among tropical popcorn germplasm. Among the seven evaluated populations, Viçosa C4 was the only one that did not show significant asymmetry between direct and reciprocal crosses. Although UCI and maternal haploid induction involve distinct biological processes, UCI acts at the pre-zygotic stage by preventing fertilization through pollen–pistil incompatibility, whereas maternal haploid induction occurs after fertilization through mechanisms associated with paternal genome elimination during early embryo development (Jiang *et al.*, 2022; Zhang *et al.*, 2018). Therefore, incompatibility between germplasm sources and inducers can prevent fertilization by inducer pollen, thus limiting maternal haploid production (Silva *et al.*, 2023). At the inbred-line level, lines 21-307-3, 21-1727-4, 21-1255-1, 21-1367-6, and 21-1634-5 showed strong incompatibility responses with inducer pollen, with direct-cross compatibility probabilities below 20%. These lines may represent valuable resources for future studies aimed at identifying and introgressing compatibility alleles through marker-assisted selection, with the objective of overcoming pollen–pistil barriers and enabling the application of maternal haploid induction in popcorn.

Although UCI represents an important challenge for the implementation of DH technology in popcorn, the main limitation is the lack of haploid inducers with high induction capacity. Despite the presence of genetic variation for HIR in the KHI population, selection based exclusively on this population may have limited potential, as high HIR is a complex trait controlled by multiple loci, including major genes and additional QTLs with smaller effects. Therefore, the most promising strategy for developing tropical haploid inducers would involve the incorporation of favorable haploid induction alleles from high-HIR inducer sources, combined with adaptation to tropical environments. When adapted high-HIR inducers are not available, crosses between haploid inducers and adapted non-inducer germplasm may represent an alternative approach to broaden the genetic base and introduce desirable agronomic traits. However, this strategy requires subsequent selection for haploid induction ability and may increase the difficulty of phenotypic screening due to the loss of inducer-associated markers,

such as *RI-nj*, which are commonly used for haploid identification. In this context, molecular markers linked to haploid induction loci, oil-content-based haploid discrimination, and emerging automated phenotyping approaches may provide valuable tools for accelerating the development of tropical-adapted haploid inducers.

6 CONCLUSIONS

The low mean HIR limits the use of KHI for developing highly efficient haploid inducers. We recommend crossing KHI with other haploid inducer sources or adapted non-inducer germplasm to generate transgressive segregants, enabling the selection of haploid inducers with higher HIR. We also identified popcorn lines from temperate germplasm showing UCI with inducer pollen, which may be used in future crosses to overcome pollen–pistil barriers between KHI and popcorn germplasm.

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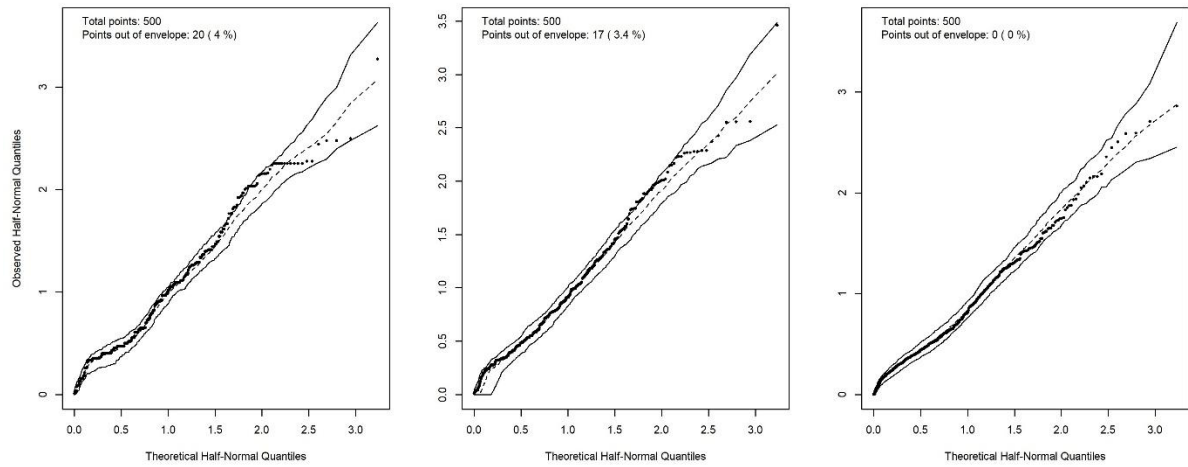
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APPENDIX A – SUPPLEMENTARY FIGURES



Supplementary Figure 1 Half-normal plots with simulated envelopes for binomial generalized linear models (GLMs) at the germplasm, population, and inbred line levels (from left to right, respectively).

APPENDIX B – SUPPLEMENTARY TABLES

Supplementary Table 1 Analysis of deviance for the effects of germplasm origin, population, inbred line, cross direction, and their interactions on cross-compatibility.

Level	Source	LR χ^2	df	P-value
Germplasm origin	Germplasm	53.93	1	<0.001
	Cross	239.51	1	<0.001
	Germplasm $\times$ Cross	24.66	1	<0.001
Population	Population	73.33	6	<0.001
	Cross	5.59	1	0.020
	Population $\times$ Cross	32.17	6	<0.001
Inbred line	Inbred line	242.52	54	<0.001
	Cross	0.24	1	0.560
	Inbred line $\times$ Cross	148.29	54	<0.001

Note: LR χ^2 , likelihood-ratio chi-square; df, degrees of freedom. Tests were based on Type III analysis of deviance.