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HETEROTIC POOLS AND REALISTIC CROSS-VALIDATION REVEAL THE USEFULNESS OF GENOMIC SELECTION IN TROPICAL MAIZE HAPLOID INDUCER POPULATIONS

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À Deus.

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ABSTRACT

The development of doubled haploid (DH) lines through *in vivo* haploid induction is a powerful tool to accelerate maize breeding. However, the process is limited by the low heritability and phenotyping complexity of haploid induction traits. This study evaluated the potential of genomic selection (GS) for improving putative (HIRp) and real (HIRr) haploid induction rates in a tropical maize population subjected to three recurrent selection cycles (C0, C1, C2), using 190 inducer progenies and testers from Flint and Dent heterotic groups. Phenotypic data were analyzed with linear mixed models, and genomic data were obtained through genotyping-by-sequencing (GBS). Heritability estimates were low (0.16 for HIRp, 0.47 for HIRr), with tester correlations of 0.11 and 0.56, highlighting the challenges of phenotypic selection. Genomic predictions with GBLUP were assessed under *k*-fold and leave-one-cycle-out (LOCO) cross-validation. LOCO consistently outperformed *k*-fold, reaching predictive ability (PA) of up to 0.79 and relative efficiency (RE) of 426 for HIRr. A key finding was the strong heterotic pool effect: although Flint sometimes presented higher PA and RE numerically, *R1-nj* marker suppression was substantially higher (46.8%) compared with Dent (0.7%). This indicates that Dent germplasm provides more reliable phenotypic inputs and therefore constitutes a more consistent foundation for GS. These results confirm that GS can significantly improve haploid induction prediction in tropical maize, particularly when validation mimics inter-cycle prediction and heterotic group differences are accounted for. The study highlights the importance of precise phenotyping, group-specific modeling, and temporal validation frameworks, reinforcing GS as a strategic tool to enhance efficiency and sustainability in DH breeding programs.

Key-words: Double haploids; haploid induction rate; Genomic prediction; *k*-fold; Leave-one-cycle-out

SUMÁRIO

1. INTRODUCTION

Maize (Zea mays L.) is one of the most economically important cereal crops worldwide, with uses ranging from food and feed to high-tech industrial applications, making it a central component of numerous productive chains. According to the Food and Agriculture Organization (FAO), global maize production reached approximately 1.16 billion metric tons in 2022, led by the United States, China, Brazil, and Argentina. For the 2024/25 growing season, total cereal production is projected at 2.85 billion tons, with maize expected to account for the largest growth by 2025/26 (FAO, 2025).

Because of its open-pollinated nature, maize breeding has traditionally relied on the development of hybrid cultivars through inbreeding and selection of superior lines (Germanà, 2011). However, the conventional development of homozygous inbred lines is time-consuming and costly, typically requiring six to eight generations of selfing (Prasanna et al., 2012). A more efficient alternative is the doubled haploid (DH) technique, which accelerates inbred line development through haploid induction (Seguí-Simarro and Nuez, 2008).

Haploid induction in maize is now a well-established tool for accelerating inbred line production, substantially reducing breeding cycle time (Prigge et al., 2012; Chaikam et al., 2019). The process involves haploid inducer lines, which, when crossed with donor plants, generate seeds with haploid embryos. These embryos are usually identified by anthocyanin-based markers such as *R1-nj* (Nanda and Chase, 1966; Geiger, 2009). In vivo haploid induction occurs when inducer lines derived from Stock6 cause the elimination of paternal chromosomes shortly after fertilization (Kelliher et al., 2017; Gilles et al., 2017). During double fertilization, one sperm nucleus fertilizes the egg to form the embryo, while the other fuses with the polar nuclei to produce the endosperm (Dresselhaus et al., 2016). The anthocyanin marker R1-navajo (*R1-nj*) produces purple pigmentation in both embryo and endosperm when there is a genetic contribution from the inducer in both tissues; in haploid seeds, only the endosperm is pigmented, enabling visual identification (Nanda and Chase, 1966; Prigge et al., 2012). Nonetheless, inhibitory alleles and environmental variation can compromise marker expression, requiring additional validation to accurately estimate haploid induction rates (Chaikam et al., 2015; Chaikam et al., 2019).

Despite advances in DH technology, its application in tropical environments remains limited, largely due to the narrow adaptability of fixed inducer lines (Chaikam et

al., 2019). In this context, breeding haploid inducer populations represents a more flexible strategy because of their greater genetic variability and broader adaptation to tropical conditions. This approach allows recurrent selection to increase the frequency of favorable alleles across successive cycles. For example, Fritsche-Neto et al. (2023) reported genetic gains of up to 63% per cycle in tropical haploid inducer populations, demonstrating the potential of this strategy compared with the slower progress typically achieved with fixed lines.

Heterogeneous populations also provide a suitable basis for genomic selection (GS), which enables the prediction of genetic values and the optimization of crossing decisions. The integration of genetic variability with marker-assisted approaches can accelerate genetic gains and improve resource efficiency in breeding programs (Almeida et al., 2020). Focusing on population improvement rather than direct inbred line development can therefore facilitate the selection of superior genotypes with greater adaptability under tropical conditions. This strategy also allows breeders to apply improvement methods such as recurrent selection with external testers, thereby accounting for heterotic groups (Fritsche-Neto et al., 2023). Recurrent selection has proven effective in improving haploid induction capacity, particularly when phenotypic data are collected across multiple selection cycles (Lashermes and Beckert, 1988; Strigens et al., 2013). However, phenotyping for both putative (HIRp) and real haploid induction rates (HIRr) is labor-intensive and time-consuming, especially when applied at scale.

Before the widespread adoption of GS, quantitative trait locus (QTL) mapping was widely used to identify genomic regions associated with complex traits such as haploid induction rate (Prigge et al., 2012; Liu et al., 2016). Although useful, QTL mapping has two major limitations: QTL effects are often population-specific and do not generalize across populations or cycles; moreover, the method tends to detect only large-effect loci, failing to capture the numerous small- to moderate-effect loci that account for much of the genetic variance. This contributes to the "missing heritability" problem (Heffner et al., 2009; Crossa et al., 2017).

Genomic selection overcomes these limitations by estimating the total genetic value of individuals from genome-wide markers, simultaneously capturing both large and small effects. Unlike QTL mapping, GS does not require prior identification of significant loci, making it more robust across diverse genetic backgrounds and selection cycles (Meuwissen et al., 2001; Crossa et al., 2010). Consequently, GS offers greater accuracy

and stability for predicting complex traits such as haploid induction capacity, supporting sustainable genetic gains in tropical maize breeding (DoVale et al., 2022).

Applying GS to tropical haploid inducer populations is a promising strategy to improve both HIRp and HIRr. Because prediction accuracy depends strongly on the validation scheme employed, selecting appropriate cross-validation strategies is essential for generating reliable and realistic estimates throughout recurrent selection cycles (Yassue et al., 2021). Comparing commonly used approaches, such as *k*-fold cross-validation and leave-one-cycle-out (LOCO), the latter of which mimics real breeding applications, can help identify effective and sustainable strategies for long-term improvement.

Traditional validation methods such as *k*-fold or reciprocal recurrent selection may overestimate prediction accuracy due to overlap between training and validation sets, violating the assumption of independence and reducing the reliability of estimates (Yassue et al., 2021). Therefore, evaluating validation strategies specifically in tropical haploid inducer populations is crucial for defining effective GS protocols, reducing computational and phenotyping costs, and enabling more reliable and economically feasible selection decisions in real-world breeding programs.

Thus, the objective of this study was to evaluate the usefulness of GS for improving both HIRp and HIRr in tropical maize.

2. MATERIAL AND METHODS

2.1 Plant material

This study was conducted using a tropical haploid inducer population developed through three cycles of recurrent selection (C0, C1, and C2). The population was derived from crosses between haploid inducer lines, including W23 and Stock6, with the goal of improving both haploid induction rate and adaptability to tropical environments (Fritsche-Neto et al., 2023). The inducer population carries the dominant *R1-nj* anthocyanin marker, enabling visual classification of haploid seeds based on embryo pigmentation. However, inhibitory alleles for *R1-nj* expression can compromise phenotypic accuracy, justifying the integration of molecular validation.

At the beginning of each cycle, 50 inducer plants were selected and crossed with two external testers representing the Flint and Dent heterotic pools. Each plant was used in five pollinations: one self-pollination (generating S1 progeny for recombination) and four induction crosses with two testers from each heterotic pool (Figure 1). A total of 190 progenies were evaluated across the three cycles (Table 1). Selection was directed toward increasing the real haploid induction rate (HIRr) while minimizing false positives in the putative rate (HIRp).

2.2 Phenotypic evaluation and statistical analysis

Phenotypic evaluation was conducted across the three selection cycles (C0, C1, and C2) using a randomized complete block design with two replications per cross. Each experimental unit consisted of two ears from the same cross. Seeds were visually classified as diploid, putative haploid (white embryo, purple endosperm), or inhibited (no pigmentation).

The HIRp and HIRr traits were calculated as:

$$HIRp = (SHP/TNS) \times 100, \text{ and}$$

$$HIRr = (RH/TNS) \times 100$$

where, *SHP* is the number of putative haploid seeds, *RH* is the number of real haploids (sum of haploids from the putative and inhibited fractions), and *TNS* is the total number of seeds. Field validation of *SHP* was conducted using a completely randomized design with four replications based on morphological traits (Chaikam et al., 2016; Melchinger et al., 2013).

An initial joint mixed-model analysis was conducted to test the significance of tester effects across the full dataset. In this analysis, genotype was treated as a random effect to enable heritability estimation. A significant inducer × heterotic pool interaction was detected, leading to separate analyses for each tester.

For individual analysis in each heterotic pool, phenotypic data were analyzed using the following linear mixed model fitted with the *breedR* package (Muñoz and Sanchez, 2020) in R (R Core Team, 2024):

$$\boldsymbol{y} = \boldsymbol{Ui} + \boldsymbol{Vh} + \boldsymbol{Sb} + \boldsymbol{e}$$

where, $\boldsymbol{y}$ is the vector of phenotypic values; $\boldsymbol{i}$ is the vector fixed effect of the inducer (gid); $\boldsymbol{h}$ is the vector fixed effect of the heterotic pool (Flint or Dent); $\boldsymbol{b}$ is the random effect of the block nested within cycle; where $\boldsymbol{b} \sim N(0, \boldsymbol{I}\sigma_b^2)$; and $\boldsymbol{e}$ is the vector of random residual effects, where $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_e^2)$. $\boldsymbol{U}, \boldsymbol{V}$, and $\boldsymbol{S}$ are the incidence matrices for $\boldsymbol{i}, \boldsymbol{h}$, and $\boldsymbol{b}$.

Best linear unbiased estimators (BLUEs) were obtained for each subset defined by heterotic pool (tester) and used in subsequent analyses.

2.3 Genomic data and quality control

Genomic DNA from inducer plants across the three cycles (C0, C1, and C2) was previously genotyped using genotyping-by-sequencing (GBS), following the protocol of Poland et al. (2012). Sequencing libraries were constructed using methylation-sensitive restriction enzymes to reduce genome complexity and enrich for low-copy regions.

The resulting SNP dataset was filtered using the following quality control criteria: call rate (CR ≥ 90%), minor allele frequency (MAF ≥ 5%), and biallelic markers, as described by de Pontes et al. (2025). Missing genotypes were imputed with the Beagle 5.0 algorithm (Browning and Browning, 2009). After quality control and imputation, the final marker dataset comprised 22,482 high-quality SNPs. Genotypes were encoded

additively (0, 1, 2) and used to construct an additive genomic relationship matrix (Ga) following VanRaden (2008):

$$\boldsymbol{Ga} = \frac{\boldsymbol{MM}^T}{2\sum p(1-p)}$$

where $\boldsymbol{M}$ is the centered genotype matrix, and $\boldsymbol{p}$ is the allele frequency vector. To improve numerical stability during inversion, a small constant (1e−5) was added to the diagonal of $\boldsymbol{Ga}$.

Marker filtering, allele frequency estimation, and genotype matrix construction were performed in R using the *SNPRelate* package (Zheng et al., 2012).

2.4 Genomic selection modeling and cross-validation methods

Genomic prediction analyses were conducted using the GBLUP model implemented in the *breedR* package (Muñoz and Sanchez, 2020):

$$\boldsymbol{y} = \boldsymbol{\mu} + \boldsymbol{Zg} + \boldsymbol{e}$$

where, $\boldsymbol{y}$ is the vector of BLUE values; $\boldsymbol{\mu}$ is the intercept; $\boldsymbol{Z}$ is the incidence matrix relating observations to genotypes; $\boldsymbol{g} \sim N\left(0, \boldsymbol{Ga}\sigma_g^2\right)$ is the vector of genomic effects; and $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_e^2)$ is the residual vector.

To assess predictive performance, two cross-validation schemes were employed:

- *k-fold (k = 3)*: he full dataset (C0, C1, C2) was randomly partitioned into three folds. Each fold was used once as the validation set, while the remaining two served as the training set. The process was repeated 30 times per fold. Stratified analyses were performed separately for the Flint and Dent pools. Because the number of individuals differed across cycles (Table 1), a balancing function was implemented to maintain cycle proportions across folds (C0 = 25; C1 = 21; C2 = 16).
- *Leave-one-cycle-out (LOCO):* Three validation scenarios were evaluated. This approach simulates practical applications of GS where the goal is to predict untested cycles:

i) C0 + C1 → C2: all progenies of the C0 and C1 cycles to predict of the C2 cycle;

ii) C0 + C2 → C1: all progenies of the C0 and C2 cycles to predict of the C1 cycle;

iii) C1 + C2 → C0: all progenies of the C1 and C2 cycles to predict of the C0 cycle.

Predictive ability (PA) was calculated as the Pearson correlation between predicted GEBVs and observed BLUE in the validation set. To evaluate the statistical significance of differences between validation methods (*k*-fold vs. *LOCO*), non-parametric comparisons were conducted using *ggstatsplot* package (Patil, 2021) with pairwise non-parametric Games-Howell test with Bonferroni correction.

Finally, the relative efficiency (RE) of genomic selection compared to phenotypic selection was computed using the equation below with based parameters time of phenotypic and genomic cycle from DoVale et al. (2022):

$$RE = \left[\left(\frac{PA}{T_{GS}}\right) \times \left(\frac{T_P}{\sqrt{h^2}}\right)\right] \times 100$$

where, PA is the predictive ability of the specific scenario of cross-validation; h^2 is the trait heritability (phenotypic) in each heterotic pool; T_P equal to 1.75 years (time per recurrent phenotypic cycle); and T_{GS} equal to 0.35 years (time per recurrent genomic cycle).

3. RESULTS

3.1 Phenotypic analysis

The joint analysis revealed significant effects for most sources of variation (Table 2) in both HIRp and HIRr. The significant heterotic pool effect suggests differential performance between testers. Moreover, the significant inducer × heterotic pool interaction indicates that the performance of the inducers varied depending on the heterotic background.

For HIRr, the effects of block nested within cycle and inducer were also significant, whereas for HIRp, only tester-related effects reached significance. Adjusted HIRp values ranged from 0 to 0.40, with a mean of 0.025. In contrast, HIRr values ranged from 0 to 0.114, with an average of 0.016.

The broad-sense heritability estimates at the entry-mean level were 0.16 for HIRp and 0.47 for HIRr. These relatively low heritability values suggest that improving these traits through phenotypic selection alone may be challenging due to substantial environmental influence and possible inducer × heterotic pool interaction.

The correlation between testers for HIRp was 0.11, while for HIRr it was substantially higher, at 0.56—indicating a fivefold increase in agreement between testers of the heterotic pools for the real induction rate. This result supports the notion that HIRr is a more stable and reliable trait for selection compared to HIRp, reinforced by its stronger predictive ability (Table 3).

3.2 Predictive ability and comparison of cross-validation methods

Across all analyses, the Flint tester generally showed higher predictive ability (PA) than Dent, with the exception of HIRp under the *k*-fold scenario (Table 3). Overall, *k*-fold validation produced lower PA values than LOCO, although statistical differences between methods were not detected.

For HIRp, significant differences between testers were observed only under *k*-fold, with Flint outperforming Dent. Among LOCO scenarios, no statistical differences were detected, but all consistently yielded higher PA values than k-fold. The C0 + C1 → C2 scenario provided the highest PA, being 12.7 times higher in Dent and 1.8 times higher in Flint compared with k-fold. Performance under LOCO was also more stable, with Flint

and Dent showing nearly identical PA values, indicating reduced tester effects for this trait.

For HIRr, Dent exhibited higher PA than Flint under *k*-fold, but this trend reversed under LOCO. In fact, the best LOCO scenario, C1 + C2 → C0, produced PA values that were 1.33 times higher for Dent and 2.7 times higher for Flint compared with *k*-fold. Although differences among LOCO scenarios were not statistically significant, their consistent numerical superiority across cycles indicates greater robustness for breeding applications.

It is important to note, however, that although Flint often achieved numerically higher PA values, these results must be interpreted with caution, as marker suppression was substantially more frequent in Flint than in Dent, which may inflate PA values due to noisier phenotypic inputs.

3.3 Relative efficiency of genomic selection

The relative efficiency (RE) of GS compared with phenotypic selection varied substantially across traits, validation methods, and heterotic pools (Figure 3).

For HIRp, the lowest RE was observed under *k*-fold for the Dent tester (20.80), indicating limited predictive value. Flint performed better under *k*-fold (161.03), but still far below LOCO scenarios. All LOCO schemes markedly outperformed *k*-fold. The highest RE was obtained in the Dent pool under the C1 + C2 → C0 scenario (263.48), which was more than 12 times higher than its corresponding *k*-fold value. For Flint, the greatest RE was observed in the C0 + C1 → C2 scenario (252.05), representing a 56% improvement over *k*-fold. Notably, HIRp predictions were relatively stable across LOCO scenarios, with Flint and Dent reaching similar efficiency levels in most cases.

For HIRr, both heterotic pools benefited strongly from LOCO. Under *k*-fold, Dent outperformed Flint (228.23 *vs.* 156.36). However, under LOCO, Flint consistently achieved higher RE, reaching 425.94 in the C1 + C2 → C0 scenario—2.7 times higher than its *k*-fold counterpart. Dent also showed clear gains, with the same scenario yielding 304.31, an increase of 33% compared with k-fold. Among LOCO schemes, the C1 + C2 → C0 scenario was the most effective overall, particularly for Flint, where the gains were the highest across all analyses.

Again, these apparent numerical advantages for Flint should be interpreted carefully, as nearly half of its seeds showed R1-nj suppression in our dataset. This likely

increased noise and artificially elevated relative efficiency estimates compared to the more phenotypically consistent Dent pool.

4. DISCUSSION

The efficiency of haploid induction remains constrained by the low heritability of induction rates and the strong phenotypic variability observed across genotypes, which compromise the accuracy of conventional selection strategies (Melchinger et al., 2016). Reported *in vivo* haploid induction rates typically range from 2% to 15% (Dönmez et al., 2023), but only 1–3% of induced seeds ultimately develop into viable double haploid (DH) lines (Prasanna et al., 2012). These limitations underscore the potential of GS as a complementary approach, particularly for low-heritability traits such as haploid induction rates. This discussion highlights factors shaping our results, including phenotypic quality, heterotic pool effects, validation strategies, and the implications of GS for tropical breeding.

4.1 Phenotypic consistency and trait quality

Double haploid technology is a powerful method for accelerating the development of homozygous maize lines, offering substantial gains in cost- and time-efficiency compared to conventional inbreeding (Trentin et al., 2020; Battistelli et al., 2013). However, the low heritability of haploid induction traits continues to represent a bottleneck (Melchinger et al., 2016). In our study, broad-sense heritability estimates were 0.16 for HIRp and 0.47 for HIRr, reflecting the inherent challenges of phenotyping under field conditions.

Importantly, heritability strongly depends on the phenotyping strategy. HIRp relies on the *R1-nj* anthocyanin marker, which acts in concert with *C1*, with R1 functioning as a bHLH transcription factor that interacts with *C1* to activate anthocyanin biosynthesis (Cone et al., 1986). The dominant *R1-nj* allele produces purple pigmentation in the scutellum and endosperm, enabling visual identification of haploids. However, its expression is inconsistent across genetic backgrounds due to inhibitory alleles such as *C1-I*, which can suppress pigmentation and lead to misclassification (Prasanna et al., 2012; Chaikam et al., 2015).

By contrast, HIRr is validated in the field using additional morphological traits, making it less vulnerable to genetic and environmental interference (Chaikam et al., 2016). This explains the stronger correlation between testers for HIRr (0.56) compared with HIRp (0.11), demonstrating the superior robustness of field-confirmed measures.

4.2 Heterotic pool effects on marker expression

The efficiency of haploid induction in tropical maize is closely linked to the reliability of the *R1-nj* marker. Its effectiveness can be compromised by transcriptional inhibitors such as *C1-I*, *C2-Idf*, and *In1-D*, which prevent pigmentation even when *R1-nj* is present (Cone et al., 1986; Chaikam et al., 2015). These alleles are disproportionately frequent in Flint germplasm and other tropical pools, while Dent germplasm typically lacks them, resulting in more consistent expression of pigmentation (Geiger and Gordillo, 2009; Röber et al., 2005; Milani et al., 2016).

Our results align with these observations. We detected only 0.7% inhibited seeds in Dent compared with a striking 46.8% inhibition in Flint, confirming that suppression is unevenly distributed across heterotic groups. Although these values were not presented directly in earlier sections, they were implicitly considered in trait computations. This sharp discrepancy mirrors findings from Silva et al. (2020) and Trentin et al. (2022), who reported higher classification error rates in Flint due to reduced or absent pigmentation.

Thus, contrary to the initial assumption that Flint testers might provide more reliable phenotyping, both literature and our dataset demonstrate that Dent testers consistently yield more robust *R1-nj* expression and higher-quality phenotype data. This reduces phenotyping noise, enhances the accuracy of GS models, and helps explain the stronger predictive performance of Dent observed in this study.

4.3 Performance of cross-validation strategies

Validation strategy is critical for estimating realistic predictive ability (PA) in GS. In our study, the LOCO approach consistently outperformed the k-fold strategy across traits and testers (Figure 2; Table 3). This distinction is especially relevant in recurrent selection programs, where breeding cycles are temporally structured.

GS accuracy depends on preserving linkage disequilibrium (LD) between markers and causal loci. Random allocation in k-fold can disrupt LD by mixing individuals from different cycles, inflating PA through shared parental effects rather than true genomic merit (Machado et al., 2023; Werner et al., 2020). LOCO better reflects intergenerational prediction by using past cycles to predict new ones, thereby maintaining LD structure and reducing overfitting (Werner et al., 2020; Yassue et al., 2021). This explains the higher PA observed in LOCO, particularly for HIRr in Dent.

Escamilla et al. (2025) emphasize that validation in cyclic breeding programs must align with the temporal logic of selection, as was achieved here. Consequently, LOCO emerges as the most realistic and practical validation framework, especially in tropical contexts where data are organized by selection cycles (DoVale et al., 2022).

Additionally, heterotic pool structure can shape LD patterns. The stronger performance of Dent observed here likely reflects both its more reliable R1-nj expression and a more homogeneous genetic architecture, reinforcing the importance of incorporating subpopulation structure in GS strategies (Escamilla et al., 2025).

4.4 Relative efficiency and implications for tropical breeding

Nonetheless, the results reported here are highly relevant for breeding programs aiming to improve haploid induction, especially under GS. The subset analyses by heterotic pool emphasize the need to consider differential suppression effects when designing breeding strategies for DH line development or open-pollinated varieties.

Our results further demonstrate that GS outperforms traditional QTL mapping for HIR and other complex traits. QTL studies in maize have generally reported low to moderate accuracies (0.20–0.40) due to the inability to capture numerous small-effect loci (Prigge et al., 2012; Liu et al., 2016). In contrast, GS in our study achieved mean accuracies of 0.46–0.66 for HIRr under LOCO validation, peaking at 0.79 in the Dent pool. This represents a two- to threefold improvement over typical QTL values, highlighting GS's ability to recover part of the "missing heritability" and deliver robust predictions across cycles.

Comparable results have been documented for other traits in maize. Technow et al. (2012) reported GS accuracies of up to 0.58 for single-cross grain yield, surpassing QTL mapping, which rarely exceeded 0.30–0.40. More recent studies also show that GS not only increases accuracy but also provides more stable predictions across backgrounds and environments (Crossa et al., 2017; Fritsche-Neto et al., 2023).

The relative efficiency (RE) of GS was particularly striking under LOCO validation, exceeding 400 for HIRr in Dent (Figure 3). This highlights GS as not only more accurate but also faster and more cost-effective than phenotypic selection. As noted by Crossa et al. (2017) and Gaynor et al. (2017), GS accelerates breeding cycles and reduces phenotyping costs, thereby increasing long-term genetic gains.

For tropical breeding programs, LOCO offers a realistic validation framework. Combined with heterotic pools less affected by *R1-nj* suppression, GS emerges as a powerful tool to streamline DH pipelines and reduce reliance on labor-intensive phenotyping (de Pontes et al., 2025; Bernardo, 2021).

It should be acknowledged that our population size was smaller than in typical GS experiments. This reflects the practical challenges of phenotyping for haploid induction, especially for HIRp, which requires labor-intensive visual screening using *R1-nj* followed by field confirmation. Although many seeds were initially classified, only a fraction were confirmed as true haploids, reducing the effective sample size. Nonetheless, the consistency of our results provides strong evidence for the utility of GS in this context.

Overall, the findings emphasize that GS can substantially improve haploid induction in tropical maize breeding, provided that heterotic group differences in *R1-nj* suppression are accounted for. In particular, the markedly lower inhibition rates in Dent highlight its strategic value for DH development and GS deployment in tropical programs.

5. CONCLUSIONS

This study demonstrated the potential of GS for improving haploid induction rate in tropical maize. Across validation scenarios, GS achieved accuracies ranging from 0.46 to 0.66 under realistic LOCO schemes, reaching up to 0.79 in specific tester–pool combinations. These accuracies represent a two- to threefold improvement compared with values typically reported for QTL mapping, underscoring the ability of GS to recover part of the "missing heritability" and generate reliable predictions across selection cycles.

The comparison of cross-validation strategies confirmed that LOCO provides more realistic accuracy estimates than *k*-fold by preserving linkage disequilibrium and respecting the temporal structure of recurrent selection programs. This validation framework avoids artificial inflation of predictive ability and is therefore the most suitable choice for practical breeding applications.

Importantly, our analyses highlighted that heterotic pools differ markedly in the expression of the *R1-nj* marker. While Flint sometimes achieved higher PA and RE in numerical terms, these values should be interpreted with caution, as 46.8% of its seeds displayed *R1-nj* inhibition, compared with only 0.7% in Dent. This high incidence of suppression in Flint compromises phenotypic consistency and can inflate prediction statistics. In contrast, Dent germplasm provided more reliable *R1-nj* expression and therefore constitutes a more robust foundation for GS-based breeding strategies.

Taken together, our findings confirm that GS not only surpasses traditional QTL mapping but also provides stable and practical predictions across breeding cycles. However, they also emphasize the need to account for heterotic pool differences in marker suppression when designing DH breeding strategies. For tropical maize programs, Dent emerges as the most strategic pool for integrating GS with DH technology. Future studies with larger populations and cost-efficient genotyping platforms may further enhance GS implementation, enabling its integration with multi-trait and environment-specific models to maximize genetic gain.

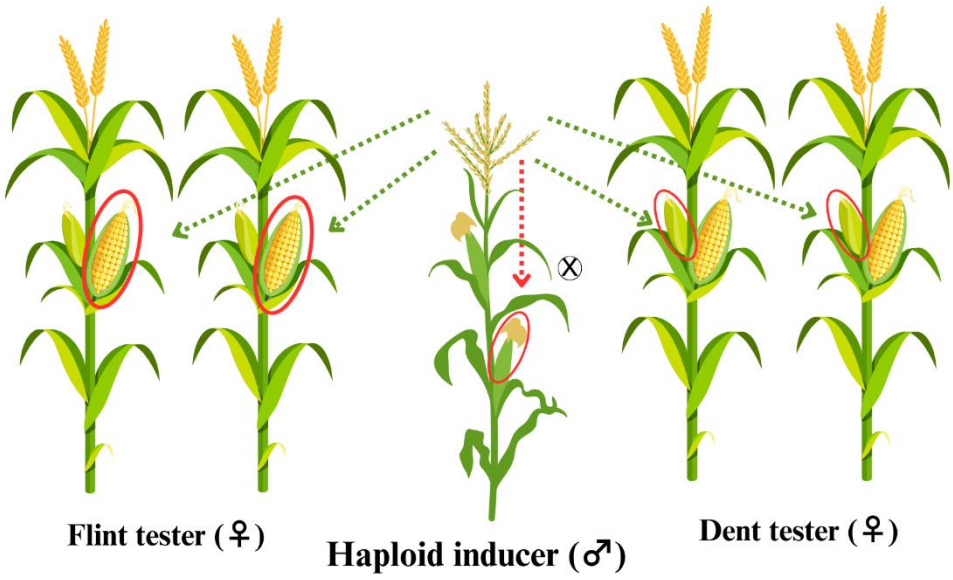

Figure 1. Crossing scheme and progeny development under intrapopulation recurrent selection with external testers (single crosses – SC). Each inducer plant was subjected to five crosses: one self-pollination and four single crosses, with two replicates for each external tester.

Adapted from Fritsche-Neto et al. (2023).

Table 1. Number of progenies derived from the tropical haploid inducer population across three recurrent selection cycles (C0, C1, and C2) and years of evaluation, including the overlap of genotypes belonging to different cycles.

Year	**Cycle**			***Sum***
	C0	**C1**	**C2**	
2018	50	-	-	***50***
2020	14	56	-	***70***
2021	15	16	52	***83***
Sum	***79***	***72***	***52***	***203***
Unique genotypes used	***77***	***63***	***50***	***190***

Table 2. Wald test for fixed effects, likelihood-ratio test (LRT) for random effects, heritability estimates (entry mean basis), variance components, overall means, and tester correlations for putative (HIRp) and real (HIRr) haploid induction rates.

Source	HIRp	HIRr
	Wald statistic	
Heterotic pool (HP)	125.80***	800.545***
	Likelihood-ratio test (LRT)	
Block/Cycle	0.783	51.586***
Inducer (I)	0.665	10.561**
I x HP	29.375***	170.091***
	Heritability and variance components	
Heritability (h^2)	0.16	0.47
$\sigma^2_{b/c}$	0	2.23×10^{-4}
σ^2_i	5.25×10^{-5}	5.88×10^{-5}
$\sigma^2_{i\times hp}$	4.07×10^{-4}	1.60×10^{-4}
σ^2_ε	8.59×10^{-4}	7.31×10^{-5}
	Overall mean (adjusted means)	
Mean	0.025	0.016
	Relationship between tester	
Correlation	0.11	0.56

Table 3. Predictive ability of genomic selection models for HIRp and HIRr traits across heterotic pool (Dent and Flint) and validation scenarios (*k*-fold and LOCO – leave-one-cycle-out). LOCO scenarios include: C0 + C1 → C2, C0 + C2 → C1, and C1 + C2 → C0.

Trait	Cross-validation method	Predictive ability		
		Dent	**Flint**	***Mean***
HIRp	*k*-fold[(1)]	0.03	0.23	***0.13***
	LOCO[(2)] - C0 + C1 → C2	0.32	0.36	***0.34***
	LOCO - C0 + C2 → C1	0.23	0.32	***0.28***
	LOCO - C1 + C2 → C0	0.38	0.20	***0.29***
	Mean	***0.24***	***0.28***	**-**
HIRr	*k*-fold[(1)]	0.39	0.29	***0.34***
	LOCO[(2)] - C0 + C1 → C2	0.47	0.54	***0.51***
	LOCO - C0 + C2 → C1	0.44	0.69	***0.57***
	LOCO - C1 + C2 → C0	0.52	0.79	***0.66***
	Mean	***0.46***	***0.58***	**-**

(1) K-fold was obtained with k = 3 folds and 30 replicates per fold.

(2) LOCO was obtained with realistic validation and for this, only observations.

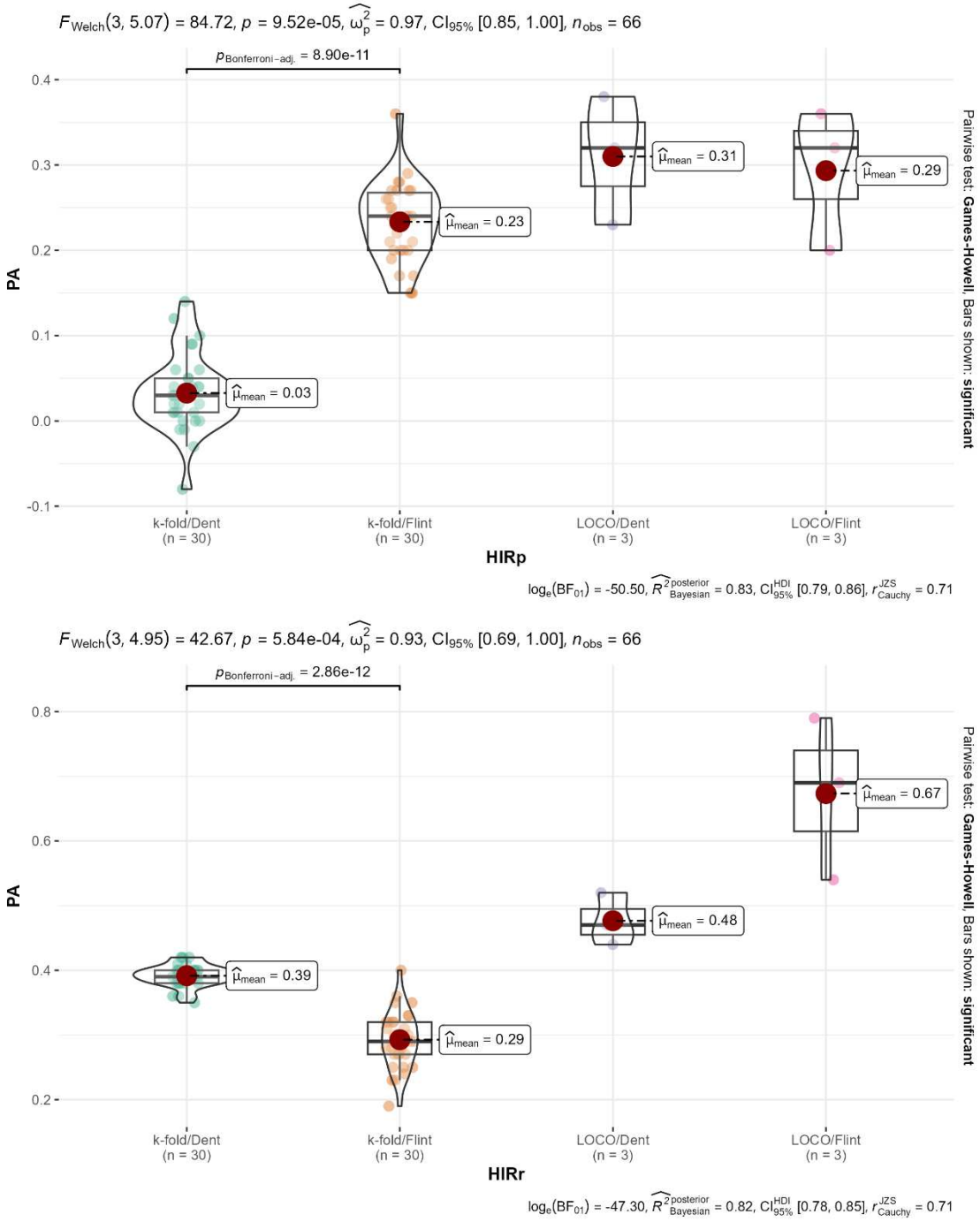

Figure 2. Distribution of predictive ability (PA) estimates for HIRp and HIRr traits under two cross-validation methods (*k*-fold with $k = 3$ and 30 replicates; LOCO with three scenarios). Dots represent individual values; dashed lines indicate group means. Statistical differences were assessed using the Bonferroni-adjusted Games-Howell test.

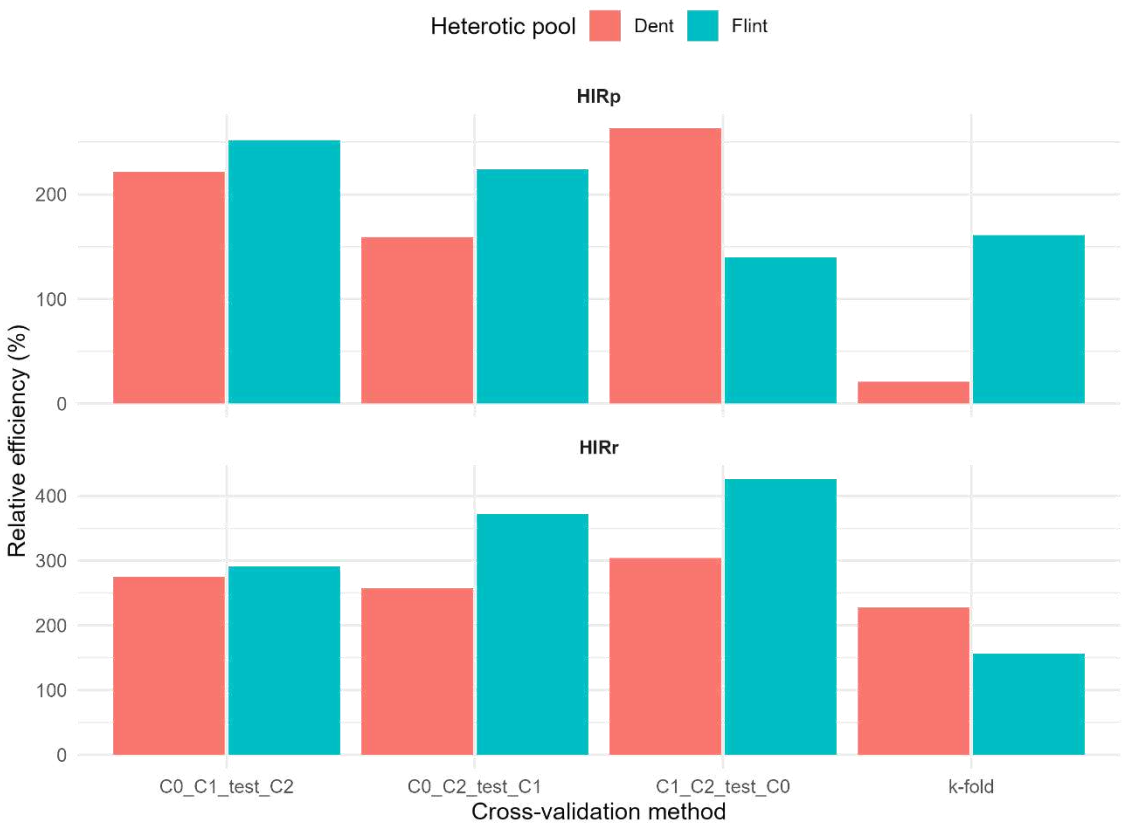

Figure 3. Relative efficiency of genomic selection compared to phenotypic selection for HIRp and HIRr under *k*-fold and LOCO validation schemes. Values are presented separately for Dent and Flint heterotic pools.

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