

Stratified haploid identification system through the *R1-nj* kernel and reduced seedling vigor in tropical maize germplasm

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Abstract. Thawarorit A, Demail A, Lertrat K, Chankaew S, Suriharn K. 2023. Stratified haploid identification system through the *R1-nj* kernel and reduced seedling vigor in tropical maize germplasm. *Biodiversitas* 24: 4262-4268. Haploid identification through the *R1-nj* marker is commonly applied in *in-vivo* doubled haploid technology in maize, although misclassification issues often occur. We explored the innate properties of maternal haploids at the seedling stage to verify true haploids and to reduce false positives. There are many questions about whether reduced seedling vigor is effective for haploid verification in tropical maize backgrounds. This study aimed to evaluate the haploid induction rate of Stock6-derived F₃ population inducers and to investigate the effectiveness of reduced seedling vigor at the V₂/V₃ stage as an alternative marker to verify putative haploids. Field trials and haploid induction were conducted in the rainy season of 2021 and the dry season of 2021/22 in Khon Kaen, Thailand. Two tropical source germplasm: P789 and S7328, were included as donor females. Haploids were identified through the *R1-nj* marker and reduced seedling vigor. Significant reduction of haploid frequency up to 2.5% on average between the two identification methods, indicating that a considerable number of false positives could be found using reduced seedling vigor. Inducer population K11 was promising since it performed a stable ability to produce haploids over donors and seasons and had a low misclassification rate. Further breeding strategies and haploid selection schemes are discussed.

Keywords: Doubled haploid technology, haploid selection, *R1-nj* marker, reduced vigor, misclassification rate

INTRODUCTION

Maize is an important cereal crop for food, feed, and fuel worldwide. The global consumption of maize grain is increasing annually, surpassing one trillion tons annually (Erenstein et al. 2022). The rising demands are followed by positive trends in global maize production, shifting from 2-ton ha⁻¹ in 1960 to 6-ton ha⁻¹ in 2020 (Erenstein et al. 2022). The remarkable increase in maize yield is attributed to using hybrid cultivars. To fully exploit the heterosis advantage, 6-8 recurrent selfings must develop sufficient homozygous inbred lines as hybrid parents. Since conventional breeding is a numbers game, thousands of inbred lines are required, and the hybrids should be tested through multi-environment trials before being available in the seed market. Therefore, to bypass the time constraint in hybrid breeding, doubled haploid (DH) technology promises to produce fully homozygous DH lines within two generations (Chaikam et al. 2019). Two protocols are available for haploid induction: *in vitro* and *in vivo*. While the *in vitro* method is more likely to be genotype- and laboratory- dependents, the *in vivo* method promises sufficient haploid production; it is more practical, making it reliable for large-scale DH production (Chaikam et al. 2019).

In the *in vivo* maternal system, haploid inducers are pollinated with female donors to produce haploids. In tropical regions, haploid inducers were lacking until, in

2018, CIMMYT released the second generation of tropically adapted inducer lines (2GTAILS) with good agronomic adaptation in tropical and subtropical environments and high haploid induction rate (HIR ~ 13.1%) (Chaikam et al. 2018). Due to either lack of access or licensing issues of available haploid inducers, most seed start-ups are lagging in adopting DH technology in their breeding programs. Besides, personal communications with private breeders agreed that the direct use of exotic haploid inducers for routine haploid induction into targeted environments is not effective due to the maladaptation syndrome of haploid inducers. There are still some weather variations within the tropics, namely tropical rainforest, savanna winter, and savanna summer. Thus, for Thailand's DH maize program, it is imperative to establish novel haploid inducers adaptive to specific regions, such as tropical savanna. One well-known public genetic stock possessing haploid induction ability is temperate inducer genotype Stock6 with 2.3% HIR (Coe 1959).

Khon Kaen University in Thailand has initiated breeding haploid inducers by introducing a temperate inducer 'Stock6' to Thai maize germplasm possessing tropical backgrounds, aiming to develop new haploid inducers performing better agronomic adaptation and HIR for tropical savanna regions (Demail et al. 2021). Positive selection gain per cycle was achieved for improving the *R1-nj* seed set, but it did not for HIR (Demail et al. 2021). The *R1-nj* kernel anthocyanin marker, a common haploid

identification system in maize, might bias the slow progress of HIR. The evidence of *R1-nj* suppression is commonly found in tropical and flint maize due to inhibitor gene *C1-l* (Chaikam et al. 2015). Other biomarkers integrated into inducers, such as the *Pl-1* red root (Chaikam et al. 2016) and kernel oil content (Melchinger et al. 2013; Melchinger et al. 2014), are suggested; however, those markers are still not available yet in our current inducer populations. Alternative visual markers regarding the innate difference between haploid and diploid seedlings are proposed. Haploid plants are less vigorous and significantly shorter (Chase 1964). Sekiya et al. (2020) applied this approach to identify haploid/diploid plants in sweet corn at V_2/V_3 stage or 10-12 days after planting (DAP) under greenhouse conditions. Most putative haploid seedlings observed showed pale leaves and green first leaf sheath. The question arises whether this approach could be adopted and effective for haploid selection in tropical maize backgrounds. Therefore, this study aimed to evaluate the HIR of Stock6-derived F_3 population inducers across two growing seasons and to investigate the effectiveness of reduced seedling vigor at the V_2/V_3 stage as an alternative marker to verify the putative haploids.

MATERIALS AND METHODS

Plant materials

Ten populations of F_3 inducers were used in this study (Table 1). These populations were established by intercrossing between temperate inducer Stock6 and three waxy corn genotypes TB, TL, and KND. Two consecutive selfing generations were performed without intense selection. Genotype Stock6 is a public inducer genotype used as the founder parent of haploid induction ability with 2.3% of haploid frequency (Coe 1959). However, this genotype had poor tropical adaptation, such as premature flowering, low fertility, susceptibility to pests and diseases, and poor seed set. Meanwhile, the Plant Breeding Research Center for Sustainable Agriculture, Faculty of Agriculture, Khon Kaen University, Thailand, developed the normal waxy genotypes. Those lines were used as the founder parent of good agronomic properties.

For *in vivo* haploid induction, two commercial hybrids S7328 and P789, were used as donor females. Both genotypes are developed by Syngenta and Pacific Seeds, respectively. Some reasons for using those genotypes as maternal donors were that they are resistant to tropical diseases, high-yielding, and large-seeded with flat embryos, facilitating haploid selection at the seed stage.

Field experiment and haploid induction

Ten inducer genotypes were laid out in a Randomized Complete Block Design (RCBD) with three replications in the rainy season of 2021 and the dry season of 2021/22 at the Agronomy Field Crop Station, Khon Kaen University (16°28'27.7" N, 102°48'36.5" E; 190 m above sea level (masl), Khon Kaen, Thailand. The plot size was five rows 5 m long, and the plant spacing was 75 × 25 cm.

Table 1. Ten populations of F_3 haploid inducers (HIs) used in this study

No.	HI genotype	Pedigree	Kernel color
1	K1	S6/TBL-2-B3	White
2	K2	S6/TBL-9-B3	White
3	K4	TLxIDL/KND10 B2-2-B4	Yellow
4	K5	S6/TBL-3-B4	White
5	K7	S6/TBL-5B-B3	White
6	K8	S6/TBL-4-B3	White
7	K9	S6/TBL-1-B4	White
8	K10	S6/TBL-7-B4	White
9	K11	S6/TBL-8-B4	White
10	K12	TLxIDL/KND10 1-B3	Yellow

Two donor genotypes were placed adjacent to the inducer plots to facilitate hand-pollination for *in vivo* haploid induction. Three staggered planting dates of donor genotypes with seven days intervals were implemented to ensure flowering synchrony. About 20 inducer plants per plot were selected and self-pollinated for inducer maintenance, and those selected plants per plot were also individually cross-pollinated to 20 ears of each donor genotype for checking haploid induction rate (HIR).

Standard agronomic practices followed the recommendations of the Department of Agriculture, Thailand, including land preparation, fertilization, irrigation, pest, disease, and weed controls.

Testing regimes for measuring haploid induction rate

All seeds from each donor's ear were first classified based on the *R1-nj* anthocyanin kernel marker on the crown (top endosperm tissue) and embryo scutellum (Nanda and Chase 1966). The putative diploids show purple colorations of the endosperm and embryo, whereas putative haploids show purple endosperm and colorless embryos (Figure 1A). Haploid induction rate (HIR) and inducer seed set (ISR) were then calculated as follows:

$$\text{HIR (\%)} = H / T \times 100$$

$$\text{ISR (\%)} = (\text{seed number of inducer expressing } R1\text{-}nj / \text{total seed number per inducer ear}) \times 100$$

Where: H is the number of putative haploid seeds based on the *R1-nj* marker, and T is the total number of seeds per tester ear.

The estimation of HIR regarding the *R1-nj* marker was called initial HIR. Therefore, to prevent potential over-estimated HIR due to high false positives through the *R1-nj* marker, the reduced seedling vigor (RSV) was observed among putative haploid seedlings. The putative haploid seedlings were planted in the plug trays and visually observed at V_2/V_3 stage or 10-12 DAP (Sekiya et al. 2020). Haploid individuals are recognized by shorter plants, narrow, erect, and light green leaves (Chase 1964). In our induction populations, haploid seedlings also show lower root densities than the diploids (Figure 1B). The misclassification rate (MCR) was then calculated as follows:

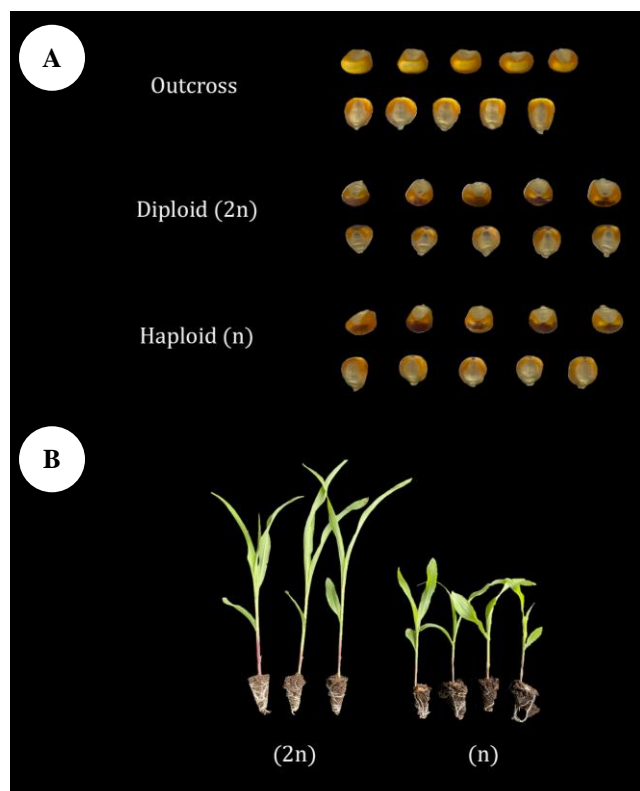


Figure 1. Two testing regimes for measuring haploid induction rate (HIR). A. The *R1-nj* anthocyanin kernel marker. B. Reduced seedling vigor observed at V2/V3 stage

$$\text{MCR} = \text{FP} / \text{HS}$$

Where FP is false positive, as the number of true diploid seedlings through the RSV test, previously classified in putative haploid class, HS is a haploid sample, as the number of haploid seeds for the RSV test.

Then, the corrected HIR (%) can be estimated as follows:

$$\text{Corrected HIR (\%)} = H (1 - \text{MCR}) / T \times 100$$

Statistical analysis

All observed data were subjected to Bartlett's test for homogeneity of variance and the Shapiro-Wilk test for normality. Then, the combined analysis of variance in RCBD multi-season was performed. Linear correlation and regression analyses were carried out.

RESULTS AND DISCUSSION

Analysis of variance

The effects of season (S), genotype (G), and the interaction between genotype and season ($G \times S$) were significant for all observed traits (Table 2). For the inducer seed set (ISR), genotype was the major source of variation. While season contributed the largest proportion of variation for corrected HIR with tester genotypes P789 (CHIR_P) and S7328 (CHIR_S), both genotype and $G \times S$ had the

largest distributions for initial HIR with tester genotypes P789 (HIR_P) and S7328 (HIR_S).

The significance of genotype indicated the diversity of ten populations of haploid inducers regarding the seed set and the ability to produce haploids (HIR). In this study, the seed set indicated the ratio between inducer seeds expressing the *R1-nj* marker and the total seed number per ear. As seen in Figure 2, the seed set of inducers was also attributed to different ear and seed dimensions (length \times diameter), resulting in variations in row number per ear and seed number per row. Those populations derived from intraspecific crosses between temperate inducer Stock6 and non-inducer waxy corn lines. Genotype Stock6 carried two known genes, *mtl* and *R1-nj* (Coe 1959), while waxy corn lines did not have any of those genes. It seemed that segregations among F_3 populations existed for given traits. The significant effect of season implied that the different weather profiles between the dry and the rainy seasons contributed to the overall changes of our inducer populations for given traits. As seen in Figures 4 and 5, the HIR means of ten populations in the dry season (3.67%) were higher than the HIR means in the rainy season (1.64%) with the donor female P789. Likewise, the seasonal effects can also be noticed when different donor females were used. With donor female S7328, the HIR means of all populations in the dry season (2.46%) were higher than the HIR means in the rainy season (1.33%). Previous studies noticed the remarkable effect of seasonal variations under tropical savanna where per se of haploid inducers was much better in the dry season than in the rainy season and proposed that the dry season was more suitable for *in vivo* haploid production (Sintanapardee et al. 2022).

The significant $G \times S$ effect suggested that each inducer population (genotype) might respond differently in each testing season for given traits. Using the donor female P789 for initial HIR evaluation via R1 test, genotype K11 had the highest HIR (2.58%) while genotype K12 had the lowest HIR (1.24%) in the rainy season (Figure 4 and 5). Meanwhile, in the dry season, two genotypes K7 (6.05%) and K5 (5.72%) performed the highest HIR while other two genotypes K10 (1.13%) and K1 (1.38%) showed the lowest HIR. In contrast, when the donor female S7328 was used for R1 test, genotype K2 possessed the highest HIR (2.50%) while genotype K7 had the lowest HIR (0.82%) in the rainy season. In the dry season, two genotypes K11 (7.57%) and K8 (7.04%) showed the highest HIR whereas the other two genotypes K4 (0.34%) and K10 (0.32%) performed the lowest HIR. The result above indicated the presence of crossover $G \times S$, and multi-environment trials are important in inducer breeding when phenotyping for those traits.

In general, the error term in Table 2 indicates that the errors may occur during conducting the experiments beyond the treatments applied. Error (a) indicates the replications within season and its mean square can be used to test the significant effect of factor season (S), while error (b) indicates pooled errors as the sum of individual error of two seasons and its mean square can be used to test the significant effect of factor genotype (G) and the interaction between genotype and season ($G \times S$). Both error (a) and

error (b) have no limitations; however, the lower MS values of both errors are favorable because we expect that the phenotypes of traits are solely caused by the treatments applied instead of the experimental errors. As seen in Table 2, the MS values of both errors were the lowest among other factors for all traits observed.

The CV (%) (a) indicates the degree of precision for the replications within season, while the CV (%) (b) indicates the degree of precision for genotype (G) and the interaction between genotype and season ($G \times S$). Both values of CV have no limitations, but the lower CV is preferred. Besides, the thresholds of CV are trait dependent. All traits observed in our study had relatively low CV, ranging from 2.0% to 14.3% for CV (a) and from 4.9% to 13.9% for CV (b), implying that the data was reliable for field experiment.

Factors affecting haploid induction (HIR) and misclassification (MCR) rates

Two testing regimes and two donor testers were assayed to confirm the actual haploid induction rate (HIR) among ten haploid inducer populations. Those two testing regimes were the *R1-nj* anthocyanin kernel marker (R1) and reduced seedling vigor at V_2/V_3 stage (RSV), while two donor testers were commercial hybrids P789 and S7328.

When the HIR was estimated based on the R1 test, the relationship between the two donors was not significant, as indicated by either regression or correlation coefficients (Figure 3A). It suggested a discrepancy in HIR when different donors were used. The difference in average HIR of ten inducers between two donors was significant, showing that donor genotype P789 had higher haploid inducibility than donor S7328 (Figure 3B). On the contrary, the RSV test showed a significant correlation between two donors for haploid inducibility (Figure 3C). The RSV-based HIR comparison between the two donors was not significantly different, implying that the actual haploid inducibility derived from both donor genotypes was similar (Figure 3C and 3D). Besides, we noticed that the corrected HIR of both donors via the RSV test was lower than the initial HIR via the R1 test, up to 2.5% of the gap, indicating

that many false positives could be found using this testing method.

Over two seasons, each of the ten inducer populations had variations of both HIR and MCR. In the rainy season, inducer genotype K11 showed the highest HIR ($>2.5\%$) and the lowest MCR ($<40\%$) when tested with donor P789. This genotype still performed high HIR ($>1.5\%$) and the lowest MCR ($<40\%$) when a different donor, S7328, was used. It indicated that genotype K11 was quite stable for HIR ability over two donors. However, other inducers showed unstable HIR with relatively high MCR ($>60\%$); even a few of them, for instance, genotypes K4, K10, and K12, had nearly 100% of MCR.

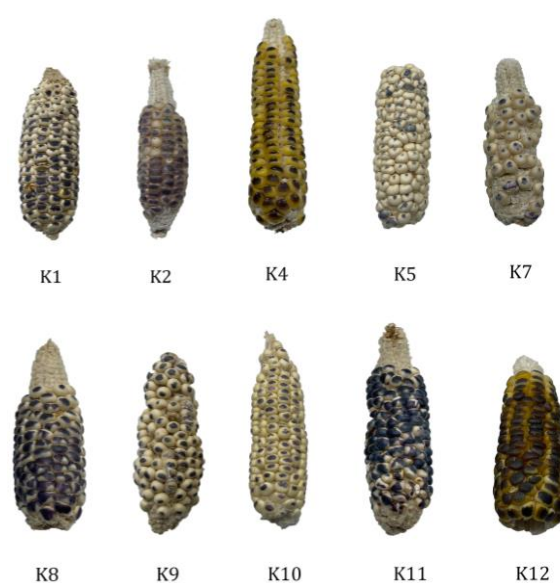


Figure 2. The ear appearance of ten inducer populations differing in the size dimensions (length \times diameter), kernel colors, and the intensity of *R1-nj* marker expression

Table 2. Mean squares of ten inducer populations for haploid induction rate (HIR) and inducer seed set (ISR) across two growing seasons between 2021 and 2022

SOV	df	HIR_P	CHIR_P	HIR_S	CHIR_S	ISR
Season (S)	1	32.63** (18.8)	2.99** (60.6)	19.2** (8.7)	3.11** (59.4)	1,620** (16.1)
Error (a)	4	0.02	0.01	0.07	0.004	0.06
Genotype (G)	9	6.84** (35.4)	0.13** (24.6)	11.9** (49.0)	0.11** (19.5)	315** (50.5)
$G \times S$	9	8.41** (43.5)	0.07** (13.0)	10.1** (41.2)	0.12** (19.9)	270** (24.2)
Error (b)	36	0.11	0.002	0.05	0.001	34.45
CV (%) (a)		6.4	14.3	13.8	12.8	2.0
CV (%) (b)		13.9	8.1	12.0	7.6	4.9

Note: The number within the parentheses is the percentage of sum of squares to the total sum of squares. HIR_P and HIR_S are haploid induction rates based on the *R1-nj* anthocyanin kernel marker with donor genotypes P789 and S7328, respectively. CHIR_P and CHIR_S are corrected HIR based on reduced seedling vigor at V_2/V_3 stage. ISR is an inducer seed set. *, ** significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. ns: not significant

In the dry season, when tested with donor S7328, two inducer genotypes, K8 and K11, had the highest initial HIR (>7%) among other inducer genotypes. Although those two genotypes retained high MCR (>80%), the corrected HIR was still the highest (>1%) among others (<1%). Excluding genotypes K8 and K11, the other eight genotypes showed remarkable shifting on initial HIR when different donors were assayed. For example, genotype K5 was top two regarding the R1 test with donor P789, but it was bottom three based on the R1 test with donor S7328. However, both per se of inducer genotype and genotypic variations for corrected HIR significantly declined; either P789 or S7328 was assayed.

The above result showed the utility of the RSV test to reduce false positives derived from the R1 test. We proposed a stratified method for haploid identification in maize in which the RSV test served as a subsequent test after pre-haploid selection via the R1 test. If the R1 test alone was used, a considerable number of false positives would be carried through the following steps in doubled haploid (DH) program that would further increase the workload, for instance, treating colchicine for haploid genome doubling and transplanting the treated haploid seedlings in the field.

The highest haploid frequency of modern haploid inducers across donor populations is still below 30% (Liu et al. 2016). The small number of haploids among undesirable hybrid kernels requires a particular ploidy discrimination method which is quick and accurate. The *R1-nj* is the R1 regulatory gene's dominant allele that regulates kernel anthocyanin biosynthesis (Petroni et al. 2014; Luo et al. 2022; Wu et al. 2022). The phenotypic expression of *R1-nj* is characterized by purple coloration in the endosperm's aleurone and the embryo's scutellum (Nanda and Chase 1966). This allele is integrated into most available haploid inducers to date, and when crossed with source germplasm lacking anthocyanin color markers, all hybrid kernels will express *R1-nj* dominance. Thus, it simplifies haploid identification at the kernel stage in maize without sophisticated equipment. However, it has some

limitations, such as being laborious, time-consuming, and having a high misclassification rate. We noticed that one trained labor could only separate the maize seeds at the rate of six to ten ears per hour. The speed and accuracy, however, depend upon the kernel *R1-nj* expression, seed set, and labor skill. The expression of *R1-nj* could be biased by two factors: the morpho-physiology of seeds and the gene \times gene interactions.

For the first factor, the seed shape, the seed moisture content, and the presence of air pockets underneath the pericarp (Prigge et al. 2011) have been reported to alter kernel *R1-nj* expression. The flat seed makes the scutellum of the embryo more visible than round seeds (Trentin et al. 2022). Delaying harvest time and extending seed drying reduce seed moisture content and create air pockets, resulting in poor *R1-nj* expression. In our study, more intense expression of *R1-nj* was likely to happen when the seed moisture content was still moderate (data not shown), but it complicated seed cracking. For the second factor, the C1 anthocyanin regulatory locus suppresses the *R1-nj* expression (Chaikam et al. 2015). Previous studies reported varying levels of *R1-nj* suppression due to inhibitor *C1-I* gene, from partial to complete inhibition among tropical maize germplasm (Prigge et al. 2011; Chaikam et al. 2015). We assumed that those reasons may explain the evidence of high MCR among inducer populations in two tropical donors (Figures 4 and 5).

We also noticed that *R1-nj*-based HIR was donor dependent. It was higher in donor genotype P789 than in donor S7328 (Figure 3). The difference may be due to seed type factor whereby genotype P789 is dent maize while genotype S7328 is flint maize. Our finding corroborated previous studies in temperate backgrounds that dent maize had higher HIR than flint maize (Trentin et al. 2022). Lower HIR in flint maize was caused by the higher accumulation of the inhibitor *C1* gene, resulting in the difficulty of haploid selection (Trentin et al. 2022), the poor intensity of the *R1-nj* marker, and high MCR (Melchinger et al. 2014).

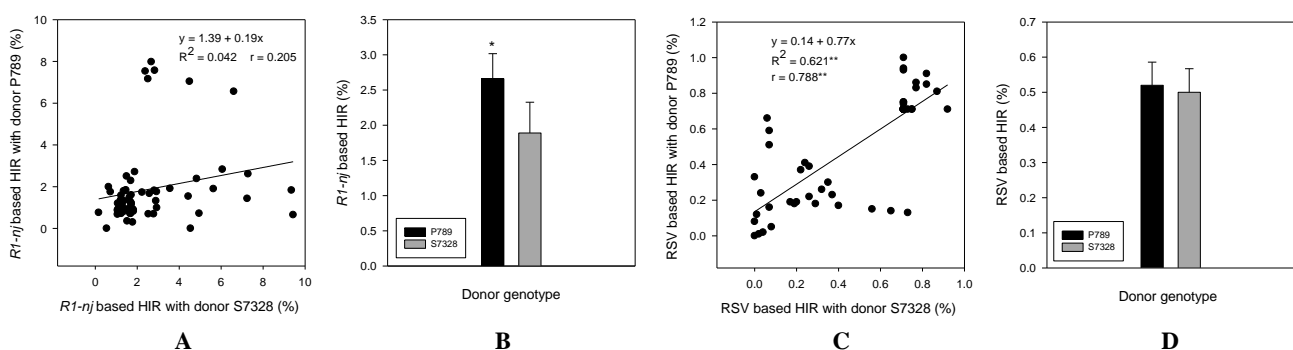


Figure 3. The association between two haploid identification systems, *R1-nj* anthocyanin kernel and reduced seedling vigor (RSV), over two donor genotypes (S7328 and P789). A. Linear regression between *R1-nj* based HIR with donor S7328 (%) and *R1-nj* based HIR with donor P789 (%). B. The means of *R1-nj* based HIR (%) in two donor genotypes. C. Linear regression between RSV based HIR with donor S7328 (%) and RSV-based HIR with donor P789 (%). D. The means of RSV-based HIR (%) in two donor genotypes

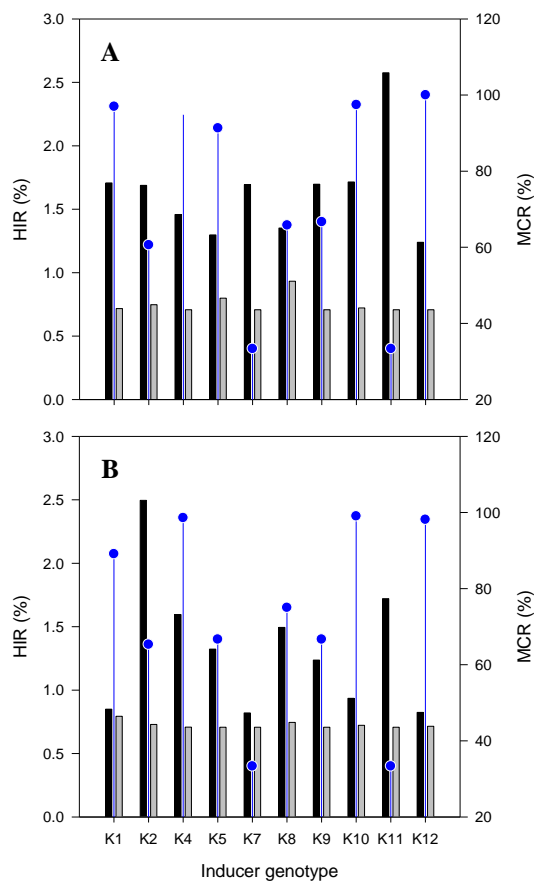


Figure 4. Haploid induction rate (HIR, %) and misclassification rate (MCR, %) of each inducer genotype determined through *R1-nj* anthocyanin kernel (black bar) and reduced seedling vigor (grey bar) with the donor genotypes P789 (A) and S7328 (B) evaluated in the rainy season of 2021. The blue dots indicate the MCR of the respective inducer genotype

Misclassification rate (MCR) was negatively correlated with embryo coloration (Prigge et al. 2011), meaning that intense *R1-nj* expression led to lower MCR. Besides, the *R1-nj* expression was transferable under additive effect (Dermail et al. 2023), implying that the intensity of expression from inducer to donor kernels will be similar. In our study, inducer K11 had intense *R1-nj* expression (Figure 1) and low MCR in the rainy season (Figure 4). Perhaps intense *R1-nj* residing in genotype K11 was also expressed well in the donors, facilitating haploid identification through the *R1-nj* marker and preventing high MCR.

Improving haploid induction rate and the reliability of haploid selection

Inducer population K11 was promising, as indicated by intense *R1-nj* expression, stable HIR, and low MCR. Besides, inducer population K8 performed good HIR in the dry season and had intense *R1-nj* expression, although there were some issues regarding high MCR and unstable HIR. We applied SNP markers on two loci responsible for HIR, *qhir1* for the *mtl* gene (Gilles et al. 2017; Kelliher et

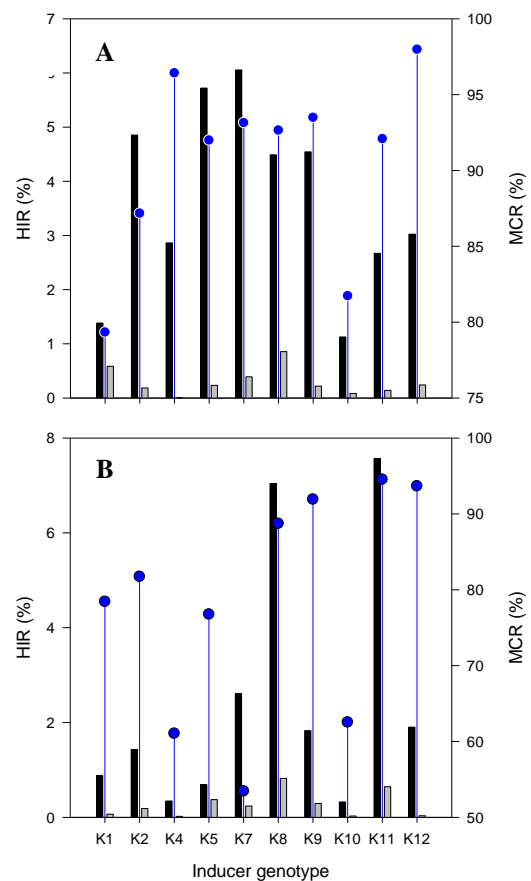


Figure 5. Haploid induction rate (HIR, %) and misclassification rate (MCR, %) of each inducer genotype determined through *R1-nj* anthocyanin kernel (black bar) and reduced seedling vigor (grey bar) with the donor genotypes P789 (A) and S7328 (B) evaluated in the dry season of 2021/22. The blue dots indicate the MCR of the respective inducer genotype

al. 2017; Liu et al. 2017) and *qhir8* for *zmdmp* gene (Zhong et al. 2019) and noticed that genotype K11 was homozygous recessive for *qhir1* only and genotype K8 was heterozygous for *qhir1*. Both *mtl* (*qhir1*) and *zmdmp* (*qhir8*) genes have the same subcellular localization expressed in the membranes of sperm cells, and act as synergistic effects. The presence of *zmdmp* will boost the haploid induction rate significantly if only the *mtl* gene is previously expressed (Zhong et al. 2019). Zhong et al. (2019) noticed that the presence of mutant *zmdmp* resulted in a 5-6-fold increase in the HIR, from 0.1-1.5% of HIR in the presence of *mtl* gene only to 7.5-10.0% of HIR in the presence of both *mtl* and *zmdmp* genes. However, our founder parent for HIR, Stock-6, only carries single *mtl* gene; thus, we could not obtain any inducer populations equipped with both *mtl* and *zmdmp* genes. To further increase their haploid induction ability, we suggested intercrossing those genotypes with elite haploid inducers fixed for both loci; for instance, inbred inducer BHI306 developed by the DH Facility of Iowa State University (DHF-ISU) (<https://www.doubledhaploid.biotech.iastate.edu/>). Then, the F_2 progenies derived from those crosses that served as base populations

will segregate for haploid induction ability. At the same time, the *R1-nj* marker may have been fixed among F₂ individuals as both founder parents have carried dominant *R1-nj* alleles. Marker-assisted selection on two loci mentioned above among F₂ individuals may fasten the selection gain since the targeted trait, HIR, is controlled by recessive alleles. Hybrid inducers are an alternative solution to enhance the efficiency of haploid induction under isolation fields by reducing the workload but still retaining the ability to produce haploids (Dermail et al. 2023; Trentin et al. 2023).

Improving the effectiveness of the haploid identification system is also imperative; otherwise, the HIR obtained will not be reliable. The use of reduced seedling vigor was effective in detecting false positives among putative haploids in tropical backgrounds. Using a similar method, Sekiya et al. (2020) could significantly reduce the false positives of haploid selection based on the *R1-nj* seed marker by up to 98% in sweet corn populations. However, Baleroni et al. (2021) reported the interaction effect between donor and ploidy levels for seedling vigor attributes, indicating the need for a specific threshold in each unique donor genotype. Therefore, further studies are encouraged to estimate the effectivity of reduced seedling vigor in wider tropical maize germplasm.

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