RESEARCH

Doubled Haploids in Tropical Maize: I. Effects of Inducers and Source Germplasm on in vivo Haploid Induction Rates

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ABSTRACT

The adoption of the doubled haploid (DH) technology in tropical maize (*Zea mays* L.) breeding programs is lagging behind that of temperate programs due to a lack of tropical haploid inducers and reliable information on the performance of temperate inducers under nontemperate conditions. The objective of this study was to determine the in vivo haploid induction ability of three temperate inducers crossed to a diverse set of tropical maize source germplasm under tropical conditions. Three experiments were conducted employing inducers as male parents to pollinate 120 source germplasm in three environments in Mexico. Haploid induction rates (HIR) obtained under field conditions were determined with two different haploid identification systems. Highly significant genotypic differences were detected among inducers and source germplasm for HIR but no interactions were observed between the two factors. Mean HIR under tropical conditions were similar to those reported for evaluations under temperate conditions indicating that temperate inducers can be employed for initiation of DH breeding programs in the tropics. Misclassification of diploids as haploids resulted in inflated HIR, particularly in highly variable source germplasm such as landraces or when expression of the identification marker was weak. We conclude that induction of haploidy is not a limiting factor for DH line production in tropical maize, but there is a need for the development of well-adapted tropical inducers.

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Abbreviations: AF08A, Agua Fría winter season 2007/2008; AF09A, Agua Fría winter season 2008/2009; CIMMYT, International Maize and Wheat Improvement Center; CML, CIMMYT maize line; DH, doubled haploid; EDC, endosperm coloration; EMC, embryo coloration; HIR, haploid induction rate; IITA, International Institute of Tropical Agriculture; *lglg,* liguleless; MCR, misclassification rate; OPV; open-pollinated varieties; QTL, quantitative trait loci; TL08B, Tlaltizapan summer season 2008.

The in vivo haploid induction technique allows breeders to develop homozygous inbred lines from heterozygous source germplasm of maize in less than half the time required with recurrent self-pollination. In addition, DH lines present several quantitative genetic, operational, logistic, and economic advantages (Geiger, 2009). The production of DH lines involves four basic steps: (i) inducing haploidy by pollinating source germplasm with pollen of a haploid inducer; (ii) identifying seeds with haploid embryo using a suitable marker; (iii) doubling the chromosomes of putative haploids with a mitotic inhibitor; and (iv) selfing DH plants to increase seed of the newly generated lines. In many maize breeding programs, biparental crosses between elite inbreds from the same heterotic pool are used as source germplasm for DH line development. In addition, landraces or open-pollinated varieties (OPV), which represent an immense reservoir of

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untapped genetic diversity (Reif et al., 2005, 2006; Beyene et al., 2006; Warburton et al., 2008), could serve as source germplasm. Doubled haploid lines extracted from landraces should also be very suitable for studying marker-trait associations because their population structure is expected to be much closer to linkage equilibrium than that of DH lines derived from elite breeding populations.

A key element for successful application of in vivo haploid induction in maize research is the production of haploid seeds from the source germplasm. Thus, a high HIR of the haploid inducer genotype is crucial. Studies in temperate maize revealed significant effects of inducers and source germplasm on HIR (Chase, 1952; Lashermes and Beckert, 1988; Eder and Chalyk, 2002; Röber et al., 2005). While Eder and Chalyk (2002) found no effect of variable growing conditions of an inducer on its HIR, Röber et al. (2005) reported a wide range of HIR across different induction environments.

Haploid inducers reported so far have been developed from temperate maize germplasm and have been mainly evaluated in temperate environments (Lashermes and Beckert, 1988; Shatskaya et al., 1994; Eder and Chalyk, 2002; Röber et al., 2005; Zhang et al., 2008). Lack of information on the haploid induction ability and agronomic performance of temperate inducers under nontemperate conditions has hitherto hampered the initiation of DH line development in subtropical and tropical (hereafter referred to as tropical) maize on a large scale. Information on the HIR of widely used temperate inducers in combination with different tropical source germplasm in various tropical induction environments will facilitate adoption of the DH technique by tropical maize breeding programs. The objectives of this study were to (i) determine the in vivo haploid induction ability of three temperate inducers under tropical conditions in Mexico, (ii) compare the response to haploidy induction shown by diverse source germplasm comprising elite single crosses, landraces, and OPVs of tropical origin, and (iii) discuss the implications of the results of this study on practical aspects of DH line development for tropical maize breeding programs.

MATERIALS AND METHODS

The study involved three temperate haploid inducers as pollen parents. These were inbreds RWS and UH400 developed at the University of Hohenheim, Germany, and their single cross RWS×UH400. Röber et al. (2005) developed RWS from a cross between inducer synthetic KEMS (Shatskaya et al., 1994) and inbred WS14 (Lashermes and Beckert, 1988), while UH400 was derived directly from KEMS (W. Schipprack, personal communication, 2010). The inducers are adapted to the temperate environments of Central Europe. They carry the dominantly inherited marker gene *R1-nj* characterized by purple coloration of the scutellum and the aleurone of seeds (Nanda and Chase, 1966; Neuffer et al., 1997), which can be used as embryo and endosperm marker, respectively, to identify putative haploid seeds. Furthermore, both inducers carry a dominant purple stalk marker (Neuffer et al., 1997) which, in the late seedling stage, facilitates the detection of "false positives" among putative haploid seeds that have been identified using the *R1-nj* marker.

Field experiments were conducted at the experiment stations of the International Maize and Wheat Improvement Center (CIMMYT) at Agua Fría (AF; 20° N lat, 104 m elevation) and Tlaltizapan (TL; 19° N lat, 930 m elevation) in Mexico during winter 2007/2008, summer 2008, and winter 2008/2009 seasons (Table 1). In all experiments, rows were 4.5 m long at Agua Fría and 5 m long at Tlaltizapan with intrarow spacing of 25 cm and interrow spacing of 75 cm. Maximum number of plants per row was 19 at Agua Fría and 21 at Tlaltizapan. Standard agronomic practices for growing maize were followed at all sites.

Experiments 1 and 2: Effects of Inducers and Environments

The effects of the inducers RWS, UH400, and RWS×UH400 were investigated at Tlaltizapan in summer 2008 (TL08B) and Agua Fría in winter 2008/2009 (AF09A). The HIR were determined based on two different haploid identification systems: the *liguleless* marker carried by a specific genotype used as female parent (Exp. 1) and the *R1-nj* color marker inherited from the inducers (Exp. 2). In Exp. 1, the single cross PDH8×PDH3 (hereafter referred to as "*lglg* tester") obtained by crossing two temperate inbred lines carrying the recessive mutation *liguleless* was used as female parent. The *lglg* tester allowed clear distinction between haploid and diploid seedlings. Haploid seedlings are characterized by absence of the ligule and auricle combined with an upright-positioned leaf enveloping the shoot (Neuffer et al., 1997). Thirty-six rows of the *lglg* tester were grown each season. The *lglg* plants were pollinated with the three inducers using a randomized complete block design with two replications. Each plot consisted of six rows of the *lglg* tester. All seeds harvested from the *lglg* plants crossed with the inducers were grown in the greenhouse until the four-leaf stage and were visually rated for the *liguleless* phenotype, an indication of haploidy.

In Exp. 2, the expression of embryo and endosperm coloration, which is caused by the *R1-nj* marker carried by the inducers, was used to identify haploids. The pollen of three inducers was used to pollinate 31 elite single crosses. These source germplasm represented elite breeding materials from the tropical breeding programs of CIMMYT in Mexico, Colombia, and Kenya, as well as the International Institute of Tropical Agriculture (IITA) in Nigeria. The experiment was laid out as a split-plot design with two replications in two environments. Source germplasm were assigned to main plots and inducers to subplots. Each main plot consisted of three rows representing one-row subplots. Thus, pollen of each inducer was used to pollinate plants of one-row subplots in each main plot. The inducer plants were grown in a field adjacent to the experiment. Twenty source germplasm were evaluated in each season. Nine of these were included in both seasons while the remaining 11 source germplasm differed so that a total of 31 source germplasm was evaluated in Exp. 2. The seed harvested from each subplot was visually scored for purple endosperm and embryo coloration using the *R1-nj* marker to identify seed with haploid embryo. Seed with purple endosperm (indicating a regular triploid endosperm resulting from successful fertilization with inducer pollen) but colorless embryo (indicating a haploid embryo of solely maternal origin) were designated as putative haploid seed.

Table 1. Information on haploid inducers, source germplasm, and environments used for Exp. 1, 2, and 3.

		Source germplasm			
Experiment	Inducers	Single cross	Landrace	OPV	Environments in Mexico
1	3†	1‡	~	~	Tlaltizapan summer 2008 (TL08B) Agua Fría winter 2008/2009 (AF09A)
2	3†	31	~	~	Tlaltizapan summer 2008 (TL08B) Agua Fría winter 2008/2009 (AF09A)
3	1§	30	29	30	Agua Fría winter 2007/2008 (AF08A)

†Two inducer inbred lines (RWS, UH400) and their single cross (RWS×UH400).

‡Single cross hybrid homozygous for the *liguleless* trait (*lglg* tester).

§Inducer inbred line UH400.

Experiment 3: Effect of Source Germplasm

Eighty-nine genetically diverse germplasm comprising 30 elite single crosses, 29 landraces, and 30 OPVs of white and yellow grain colors, and flint, dent, and semi-flint/semi-dent grain types were used as source germplasm in this experiment. The single crosses and OPVs represented elite breeding materials from the tropical breeding programs of CIMMYT in Mexico, Colombia, and Zimbabwe, and of IITA in Nigeria. The landraces were obtained from CIMMYT's germplasm collections in Mexico. They included accessions from various altitudes in 17 countries: five from the Americas, nine from Africa, and three from Asia. The experiment was conducted during the 2007/2008 winter season in Agua Fría (AF08A). Each source germplasm was grown in four-row plots using a randomized complete block design with two replications and was pollinated with pollen of plants of the haploid inducer line UH400 planted adjacent to the experiment. Putative haploids among the seeds harvested from inducer by source germplasm crosses were identified as described in Exp. 2.

Traits Assessed

The intensity of purple coloration of endosperm (EDC) and embryo (EMC) was scored visually using a scale of 1 to 5 (1 = intense coloration, 5 = no coloration) on a sample of approximately 100 seeds of each plot in Exp. 2 and 3. To verify the putative haploids and examine the effectiveness of the *R1-nj* color marker for the identification of haploids, a sample of 100 putative haploid seeds from each of the 89 source germplasm of Exp. 3 was grown in the field at Tlaltizapan during the summer of 2009. The plants were visually scored at the preflowering stage for stalk coloration and other plant characteristics: haploid plants were characterized by no purple stalk coloration as well as shorter stature, thin stalk, and erect and narrow leaves. Based on this, the misclassification rate (MCR) was computed for each source germplasm as the number of plants with purple stalk coloration divided by the total number of putative haploid seeds planted, assuming that nongerminated seeds were haploids. In Exp. 1 and 2, the haploid induction rate of each inducer was computed on a plot basis as $\mathrm{HIR} = \frac{\mathrm{H}}{\mathrm{T}}$, where H is the number of putative haploid plants (Exp. 1) or seeds (Exp. 2) and T is the total number of plants or seeds monitored. In Exp. 3, HIR were corrected for MCR as $\mathrm{HIR} = \frac{\mathrm{H}(1 - \mathrm{MCR})}{\mathrm{T}}$, where H and T are as explained above.

Statistical Analyses

Angular transformation was used to normalize distribution of residuals and remove heterogeneity of error variances of the HIR data. For missing data, iteratively computed values with least squares procedures were used (Yates, 1933; Healy and Westmacott, 1956). Data generated in Exp. 1 were analyzed using the statistical model $y_{ijk} = \mu + g_i + e_j + ge_{ij} + r(e)_{kj} + \varepsilon_{ijk}$, where μ = overall mean, g_i = effect of inducer i, e_j = effect of environment j, ge_{ij} = effect of interaction between inducer i and environment j, $r(e)_{kj}$ = effect of replication k within environment j, and ε_{ijk} = effect of experimental error for the plot containing inducer i in replication k within environment j. Except for the random error term, all effects were considered fixed.

The data generated in Exp. 2 were first analyzed individually for each of the two environments for 20 source germplasm using the model $y_{ijk} = \mu + s_i + g_j + sg_{ij} + r_k + \varepsilon_{ik} + \varepsilon_{ijk}$, where μ = overall mean, s_i = effect of source germplasm i, g_j = effect of inducer j, sg_{ij} = effect of interaction between source germplasm i and inducer j, r_k = effect of replication k, ε_{ik} = effect of experimental error for the main plot containing source germplasm i in replication k, and ε_{ijk} = effect of experimental error for the subplot containing source germplasm i pollinated with inducer j in replication k. For the nine source germplasm evaluated in both environments, we used the same model but extended it for the effects of the environment and its interactions with all other effects. In both models the effect of replication was considered random, while all other effects were considered fixed.

The data generated in Exp. 3 were analyzed using the model $y_{ijk} = \mu + m_i + s(m)_{ij} + r_k + \varepsilon_{ijk}$, where μ = overall mean, m_i = effect of germplasm group i, $s(m)_{ij}$ = effect of source germplasm j within germplasm group i, r_k = effect of replication k, and ε_{ijk} = effect of experimental error for the plot containing source germplasm j within germplasm group i in replication k. In this model the effects of the source germplasm within germplasm group and the experimental error were considered random and all other effects were fixed. Estimates of the genetic variance for source germplasm within germplasm groups were obtained by analyzing the data for the three germplasm groups separately with the model $y_{ij} = \mu + s_i + r_j + \varepsilon_{ij}$, where μ = overall mean, s_i = effect of source germplasm i, r_j = effect of replication j, and ε_{ij} = effect of experimental error for the plot containing source germplasm i in replication j. All statistical analyses were performed using the statistical software PLABSTAT (Utz, 2004).

Table 2. Mean squares (MS) for haploid induction rates (HIR) computed for Exp. 1 and 2 across two environments (df = degrees of freedom).

Source	Experiment 1			Experiment 2		
	df	MS		df	MS	
Inducer (I)	2	36.63	**	2	57.04	**
Environment (E)	1	21.46	**	1	0	
I × E	2	5.83	*	2	1.34	
Error main plot	–	–		16	10.91	
Source germplasm (SG)	–	–		8	408.64	**
I × SG	–	–		16	3.5	
E × SG	–	–		8	16.22	
I × E × SG	–	–		15	8.82	
Error subplot	4	0.38		35	7.12	

* Significant at $P < 0.05$.

** Significant at $P < 0.01$.

RESULTS

In Exp. 1, significant mean squares were detected in HIR for genotypes ($P < 0.01$), environments ($P < 0.01$), and genotype × environment interaction ($P < 0.05$) (Table 2). Mean HIR of the three inducers obtained with the *lglg* tester was significantly higher for the Agua Fría winter season than for the Tlaltizapan summer season. The differences among inducers were also more pronounced during the winter season (Table 3). Across the two environments, RWS exhibited the highest mean HIR followed by RWS×UH400 and UH400. Mean HIR of RWS was significantly ($P < 0.05$) higher than that of RWS×UH400 and UH400 for Agua Fría winter season, while no significant differences were observed between RWS and RWS×UH400 for Tlaltizapan summer season.

In Exp. 2, significant genotypic differences ($P < 0.01$) in HIR (Table 2) and EMC (data not shown) were observed for inducers and source germplasm. However, differences in environments or inducer × source germplasm interactions were not significant. Mean HIR across the two environments and nine source germplasm did not differ significantly between RWS and UH400 but were significantly higher for RWS×UH400. Ranking of inducers was similar for mean HIR across 20 source germplasm within each environment (Table 3).

Mean HIR for the nine source germplasm evaluated in the two environments in Exp. 2 ranged from 0 to 11.3% (Table 4) and was highest in source germplasm involving inbred CML445. No haploid seed was obtained in source germplasm no. 9. Purple color was consistently not expressed in this source germplasm across inducers and across environments.

In Exp. 3 using inducer UH400 as pollinator, we detected significant ($P < 0.01$) genotypic differences for HIR between the three germplasm groups, while among source germplasm significant differences were only detected within the group of single crosses (Table 5). Similar results were obtained for EMC (data not shown). Mean HIR obtained from crosses between UH400 and OPVs were significantly lower than those obtained from single crosses and landraces. Mean EDC and EMC was less intense in landraces (data not shown).

Table 3. Means and ranges of haploid induction rates (HIR) of three inducers determined with the *liguleless* (*lglg*) tester in Exp. 1 and with the *R1-nj* seed coloration in Exp. 2 during Tlaltizapan summer season (TL08B) and Agua Fría winter season (AF09A) as well as across the two environments (ACROSS).

Experiment & ID system	Source germplasm	Environment†	Inducer	N‡	HIR, %	
					Mean§	Range
Exp. 1 (*lglg*)	1	TL08B (11.01%)	RWS×UH400	8,890	12.43a	~
			RWS	10,073	12.22a	~
			UH400	9,037	8.38b	~
	1	AF09A (14.26%)	RWS×UH400	3,815	14.42a	~
			RWS	3,218	19.05b	~
			UH400	2,795	9.30c	~
	1	ACROSS (12.63%)	RWS×UH400	12,705	13.43a	~
			RWS	13,291	15.64b	~
			UH400	11,832	8.84c	~
Exp. 2 (*R1-nj*)	20	TL08B (8.92%)	RWS×UH400	115,854	9.97a	0–15.86
			RWS	44,856	8.87a	0–21.46
			UH400	117,075	7.93b	0–17.31
	20	AF09A (7.43%)	RWS×UH400	59,770	8.60a	0–15.83
			RWS	53,067	7.38b	0–13.19
			UH400	54,129	6.31c	0–10.68
	9	ACROSS (7.63%)	RWS×UH400	82,597	8.90a	0–13.19
			RWS	51,361	7.18b	0–11.54
			UH400	76, 471	6.80b	0–9.84

†Values in parentheses are the mean HIR obtained in the respective environment averaged across the three inducers.

‡Total number of plants or seeds scored for haploidy.

§Means were back-transformed to approximate their original values in percent; means followed by the same letter within a combination of identification systems and environments are not significantly different at $P < 0.05$ according to *t* test.

Table 4. Pedigrees of nine source germplasm, means and ranks for haploid induction rate (HIR), and ranks for endosperm (EDC) and embryo (EMC) coloration obtained after pollination of source germplasm with three haploid inducers averaged across three inducers and two environments in Exp. 2.

		HIR		EDC	EMC
No.	Pedigree of source germplasm†	Mean, %‡	Rank	Rank§	Rank§
1	P43SRC9FS100–1-1–8\#1/TZEESRW1-B1/EECOMP./Katumani/ KATUMANI45–4-1/ECA-EE-POP1-B-B-9/CML445	11.33	1	3	3
2	TZLCOMP3–168–2-5-\#/TZEESRW1-B1/EECOMP./Katumani/ KATUMANI35–11–1/ECA-EE-POP1-B-B-6/CML445	11.14	2	5	7
3	8721SR-34G-3–3sb-\#1/TZEESRW1-B1/EECOMP./Katumani/ KATUMANI23–11–2/ECA-EE-POP1-B-B-3/CML445	8.64	3	7	4
4	CML445/CML144//CML159//POOL15QPMSR-B-42-B/CML159	8.60	4	6	6
5	CarotenoidSyn3-FS8–4-3-B-B-B//(KU1409/DE3/KU1409)S2–18–2-B	7.57	5	1	2
6	CML379/CML144//CML159//POOL15QPMSR-B-50-B/CML159	7.39	6	8	8
7	CML312/CML-300-B-B//KUI carotenoid syn-FS17–3-2-B-B-B	7.31	7	4	5
8	CML451/KUI carotenoid syn-FS17–3-3-B-B//CML297	6.69	8	2	1
9	CML300-B-B-B//(KU1409/DE3/KU1409)S2–18–2-B	0.00	9	9	9

†Source germplasm no. 1 to 4 and 6 were provided by CIMMYT's Kenyan breeding programs and source germplasm no. 5 and 7 to 9 by the Mexico-based HarvestPlus breeding program.

‡Means were back-transformed to approximate their original values in percent; LSD = 2.67 ($P < 0.05$).

§Lower rank indicates better seed coloration based on a scale 1 to 5 (1 = intense coloration, 5 = no coloration).

It is evident from the MCR of the three source germplasm groups that the *R1-nj* system was most effective in single crosses and least effective in landraces in Exp. 3. In half of the crosses involving the landrace source germplasm, MCR was more than 30%, while in a quarter of these it was more than 50% (Fig. 1). No significant differences were found between mean MCR of the dent (33%) compared to the flint (34%) source germplasm. The MCR was lower when embryo coloration was more intense (EMC smaller or equal to 2, Fig. 2).

Table 5. Means, ranges, and genetic variance component estimates (VC) for haploid induction rates (HIR) obtained with inducer UH400 for the three germplasm groups of single crosses (SC), landraces (LR), and open-pollinated varieties (OPV) in Exp. 3 (df = degrees of freedom).

	Haploid induction rate				
Source†	Mean, %‡	Range, %	df	VC	
SG within SC group	4.55a	0.91–11.46	29	8.89	**
SG within LR group	4.04a	1.67–10.59	28	0.83	
SG within OPV group	2.46b	1.23–4.98	29	–0.18	
Error	–	–	85	10.69	

** Significant at $P < 0.01$.

†SG = source germplasm.

‡Means were back-transformed to approximate their original values in percent; means followed by the same letter are not significantly different at $P < 0.05$ according to *t* test.

DISCUSSION

Performance of Temperate Inducers in Nontemperate Environments

The availability of a reliable haploid inducer is a major requirement for DH line production in maize. Our results showed that RWS and UH400 have high haploid induction ability even in nontemperate environments. Mean HIR of RWS, which was developed from a cross between inducers WS14 and KEMS, was consistently higher than that of UH400, which was derived directly from KEMS. This suggests that WS14 carries additional favorable alleles for HIR and/or that epistatic interactions exist between alleles of the two parents of RWS. These findings are in agreement with the results of analyses of quantitative trait loci (QTL) for HIR. In a cross between haploid inducers Stock6 and WS23*ig*, Deimling et al. (1997) detected two QTL. Both parents carried alleles that favorably influenced HIR and there were significant epistatic effects between the two QTL. Future research should investigate the role of epistasis for the inheritance of in vivo haploid induction.

In Exp. 1 inducer RWS×UH400 showed intermediate HIR compared to its parent lines, while in Exp. 2 it showed higher HIR than both parents. Röber et al. (2005) reported intermediate HIR of an inducer single cross compared to its parents, whereas Geiger (2009) reported that HIR of another inducer single cross exceeded that of the better parent. Intermediate performance of the inducer single crosses indicates the presence of additive gene action (Falconer and Mackay, 1996). In contrast, higher HIR of the inducer single cross compared to the better parent may have been caused by positive epistatic interactions and/or segregation distortion (Barret et al., 2008). Further studies are needed to confirm these conclusions.

While HIR of temperate inducers were high, their relatively poor agronomic performance under tropical conditions is likely to impede their exploitation for large-scale haploid induction in tropical environments. Difficulties are encountered not only in the maintenance and seed increase of inducers due to susceptibility to tropical diseases, poor plant growth, and poor seed set, but also during cross-pollination with source germplasm due to poor pollen production under hot weather conditions. Further, poor pollen-silk

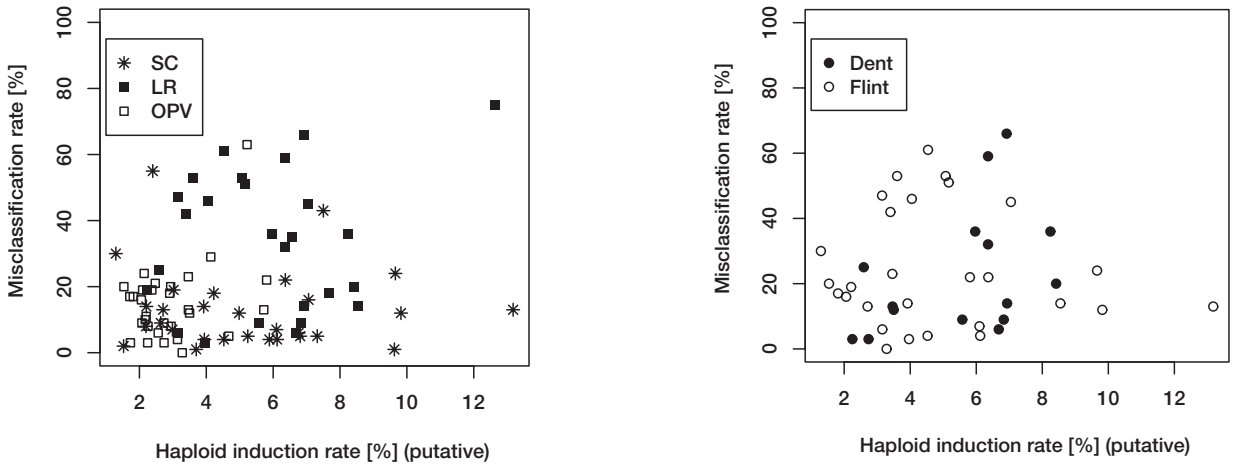

Figure 1. Misclassification rates (MCR) in relation to putative haploid induction rates (HIR) observed in crosses with inducer UH400 and 89 source germplasm in Exp. 3, separately displayed for (left) single crosses (SC), landraces (LR), and open-pollinated varieties (OPV), and for (right) dent and flint grain type source germplasm.

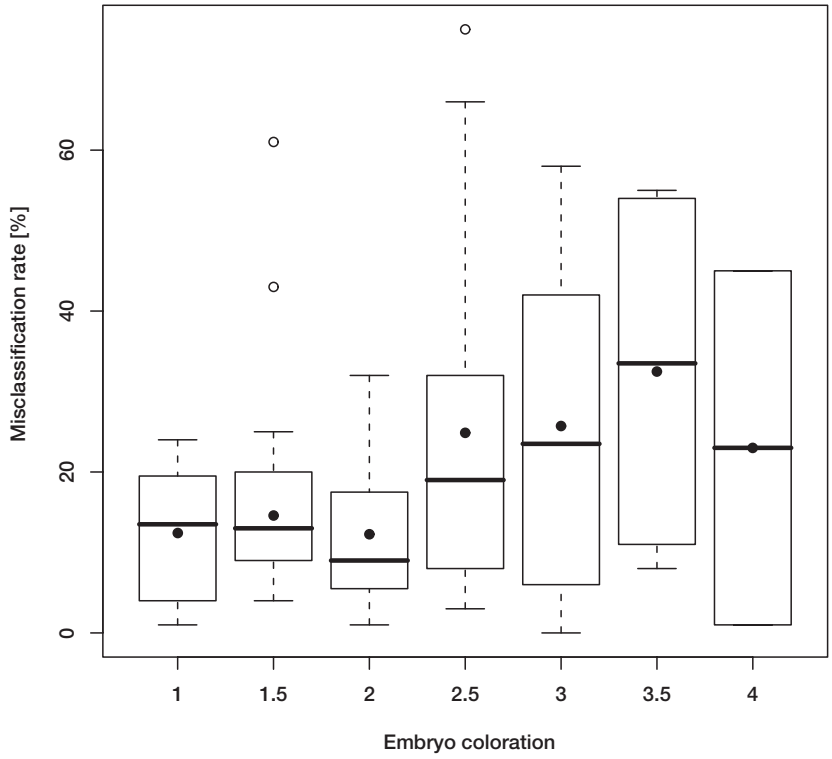

Figure 2. Boxplot with median, quartiles, and extremes of misclassification rate (MCR) displayed separately for the embryo coloration classes (scale 1–5) based on entry means of 89 source germplasm in Exp. 3 (solid circle represents the arithmetic mean of MCR in the corresponding class; classes above EMC score 4 were not observed).

synchrony of inducers and source germplasm due to their varying adaptation to different agroecologies was observed and staggered planting was necessary. In general, shading nets may help to improve production of fertile pollen. Furthermore, the relatively shorter plant height of inducer lines (often <1 m) required hand pollinations. However, efficient large-scale haploid induction would depend on reliable cross-pollination by wind in isolation blocks. In our study, RWS×UH400 exhibited heterosis for vigor and pollen production and excelled with pollen shedding for up to a week. Therefore, the use of inducer single crosses seems advantageous over inducer inbreds. As an alternative to employing temperate inducers, new inducers with better adaptation to tropical environments and acceptable HIR are currently being developed in a collaborative effort between CIMMYT and the University of Hohenheim, Germany. Furthermore, maize accessions held in global germplasm collections could be systematically screened for HIR to identify genotypes possessing haploid induction ability. In

combination with existing stocks these could be exploited to develop new inducers with substantially enhanced HIR.

Environmental Effects on Haploid Induction Ability

Significant effects of the environments on HIR of the three inducers were observed in Exp. 1, in which the *lglg* tester was employed to detect haploids, but not in Exp. 2, in which haploids were identified based on the *R1-nj* system. Earlier studies also did not show consistent environmental effects. For example, Röber et al. (2005) reported that environments strongly influenced HIR of RWS in combination with the *lglg* tester. In contrast, Eder and Chalyk (2002) found no significant effects of the growing conditions on the inducer's HIR determined with the *R1-nj* marker in combination with 12 source germplasm. These studies indicate that the results may be specific to the different haploid identification systems employed and may also depend on the genotype employed as female parent.

With the *lglg* tester, HIR was higher in the Agua Fría winter season than in the Tlaltizapan summer season in the present study. Since the effects of seasons and locations were confounded, it is difficult to identify the most important environmental factor, but temperature may have played a major role. Unlike the source germplasm used in Exp. 2, which has tropical adaptation, the *lglg* tester as well as the inducers are adapted to temperate climate and may have responded positively to cooler temperature in the Agua Fría winter season. There is a need for further studies on the effects of environments on HIR.

Effects of Source Germplasm

Significant variation for response to haploidy induction was detected among source germplasm in Exp. 2 and 3. The higher HIR obtained in single crosses compared to OPVs may be attributed, among other factors, to the more homogeneous appearance of single cross seeds which facilitated easier detection of those with haploid embryo. Within source germplasm groups, significant genotypic variation for response to haploidy induction was only found among single crosses in Exp. 3. The lack of significant variation among landraces and OPVs may be due to larger variation within these highly heterogeneous populations compared to the smaller variation between populations (Warburton et al., 2008).

The lack of significant interaction between inducers and source germplasm in Exp. 2 confirms the results of Röber et al. (2005). This may be attributed to the two inducer inbreds sharing a common parent (KEMS) and all haploid inducers reported to date tracing back to Stock6 (Coe, 1959). This indicates that, among the present inducers, any inducer with generally high HIR may be used to induce haploidy in tropical maize breeding programs.

With in vitro haploid induction methods such as anther or microspore culture (Büter, 1997), often <50% of the source germplasm develop embryo-like structures (Hongchang et al., 1991). In contrast, haploid seeds were obtained from all but two of the 120 source germplasm evaluated in Exp. 2 and 3 using the in vivo approach. Even though haploids may have been present in these two source germplasm, they could not be detected because of nonexpression of embryo and endosperm coloration. Complete inhibition of *R1-nj* encoded coloration occurs if dominant antocyanine inhibitor genes, such as *C1-I* (Coe, 1962), are present. The consistent lack of color expression in source germplasm no.9 indicates the possibility of the presence of such gene(s). Source germplasm no.9 was derived from a cross between CML300 and (KU1409/DE3/KU1409)S2–18–2-B. Both of these inbreds were also parents of other source germplasm (Table 4) which showed moderate to good EDC and EMC. Thus, neither of the two inbreds seems to be the sole carrier of the gene(s) responsible for complete suppression of coloration in source germplasm no.9. Most probably, complementary genes from both inbred parents are involved.

Haploid Identification

With haploid identification systems, as in statistical hypothesis tests, two types of incorrect decisions can be made: the haploid seed or plant can be discarded by mistake (type I error), or the normal F_1 seed or plant can be misclassified as haploid (type II error). Type I errors may have occurred due to the limited efficacy of the *R1-nj* color marker or due to insufficiently trained technical staff. Screening seeds for haploidy at the time of harvesting or immediately thereafter (before drying) may reduce this error because *R1-nj* encoded embryo coloration is usually more clearly visible at this stage. In contrast, if seeds are screened after drying, true haploids may be inadvertently discarded. During drying, sometimes air pockets develop underneath the pericarp region covering the embryos which causes the appearance of darker shades that may be wrongly perceived as embryo pigmentation. Similarly, seeds carrying a haploid embryo but exhibiting very poor endosperm coloration may be misclassified as nonpigmented seeds. These type I errors may have decreased the HIR determined with the *R1-nj* color marker as compared to those determined with the *lglg* tester. Furthermore, positive interactions between the temperate inducers and the temperate *lglg* tester may also have contributed to higher HIR in Exp. 1.

Misclassification rates, that is, type II errors, remained within an acceptable range for the majority of OPVs and single crosses, whereas in landraces a large proportion (up to 75%) of putative haploids was in fact diploid. The relatively less intense mean EMC found in landraces compared to OPVs and single crosses may have been responsible for the less accurate haploid identification and, consequently, the high MCR observed in the landrace germplasm group. The use of a larger sample of putative haploids could have facilitated a more reliable determination of MCR,

particularly in the case of source germplasm showing low MCR. A high magnitude of type II errors was reported by Röber et al. (2005) for the European flint germplasm pool, in which MCR in the putative haploid fraction selected with the *R1-nj* marker were more than 50% in many source germplasm. In contrast, the tropical flint germplasm investigated in our study did not show any such problem.

We conclude that two types of errors affect HIR determined with the *R1-nj* system but both will likely diminish over the years as favorable alleles for improved EDC and EMC accumulate with each DH breeding cycle due to selection against inhibitor genes suppressing *R1-nj* expression. Furthermore, it is expected that with time the technical staff will gain more experience in haploid identification. Nonetheless, alternative dominantly inherited marker systems should be developed. These new systems could render haploid identification more efficient and perhaps even facilitate automatization. Additionally, they may enable haploid induction in source germplasm carrying antocyanine inhibitor genes. Furthermore, new tester(s) well adapted to tropical conditions need to be developed for use in the prevailing environments to accurately monitor HIR during the development and maintenance of tropical inducers. Suitable tester germplasm carrying recessive mutants such as *liguleless* or *glossy* (Neuffer et al., 1997) may be identified in the vast collections of maize conserved in germplasm banks.

Implications for Doubled Haploid Line Development in Tropical Maize

The efficiency of maize breeding programs can be enhanced and varietal development accelerated through adoption of the DH technique assuming that resource allocation will be optimized and appropriate breeding procedures will be adopted (e.g., Seitz, 2005; Longin et al., 2006; Gordillo and Geiger, 2008). The DH technique is successfully used in many maize breeding programs in temperate areas. However, the adoption of the technique has lagged behind in tropical regions where it is only recently gaining attention due to its advantages.

Temperate inducers showed high HIR for exploitation in tropical regions. Results of our study have demonstrated that it is possible to use the available technology of in vivo haploid induction in tropical maize breeding programs. However, the adoption of the DH approach is expected to result in strong selection pressure in favor of germplasm with good response to haploidy induction and survival after artificial chromosome doubling. Problems may arise if this positive response of a particular source germplasm is associated with poor agronomic performance. For example, when an agronomically poor inbred with good response to haploidy induction is crossed with an inbred with good agronomic performance but poor response to haploidy induction, strong selection pressure in favor of positive responsiveness may be associated with selection in favor of poor agronomic performance due to linkage. Thus, allele frequencies may deviate from Mendelian segregation ratios. The occurrence of such segregation distortion could be detected with molecular markers. In general, low responsiveness of specific source germplasm can be overcome by pollinating more plants from such source germplasm to obtain the targeted number of DH lines. In conclusion, induction of haploidy is not a limiting factor for DH line production from tropical source germplasm but the development and deployment of well-adapted inducers is recommended in the long term.

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