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Germplasm enhancement of maize: a look into haploid induction and chromosomal doubling of haploids from temperate-adapted tropical sources

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With 4 tables

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Abstract

The allelic diversity (AD) project of the Germplasm Enhancement of Maize (GEM) programme utilized the double haploid (DH) breeding method to expedite development and release of lines derived from 300 exotic maize races. Using 18 races in this study, differential effects on haploid induction rates (HIRs) and doubling rates (DRs) by the recurrent parents PHB47 and PHZ51, the elevation that the race is traditionally grown at, and by the race itself were addressed in this study. Races from the AD project were grouped by elevation of their origin, high, middle or low altitude. Six races per elevation were randomly selected and backcrossed using both recurrent parents to generate 36 populations. Ten replications were randomized in a complete randomized design for two growing seasons. The recurrent parent effect was significant, with PHB47 having a higher HIR than PHZ51. Effect of elevation was significant with higher HIR associated with low-elevation origin, and race also proved to be significant. Effects of elevation, recurrent parent and race were not significant for DR.

Key words: doubled haploid — GEM — diversity — maize genetic resources

In vivo induction of maternal haploids can be accomplished by pollinating a female plant with pollen from a haploid inducer line, resulting in a small amount of haploid progeny in addition to normal F_1 progeny (Geiger 2009). There are two hypotheses on haploid embryo formation during *in vivo* induction in maize. The first hypothesis involves two sperm cells, one of which is non-functional. The non-functional sperm cell fuses with the egg cell and the normal sperm cell fuses with the central cell to create a normal triploid endosperm (Geiger 2009). Subsequently, the non-functional sperm cell's chromosomes are purged from the embryo through multiple cell divisions (Geiger 2009). The other hypothesis involves two normal sperm cells, but one cannot fuse with the egg cell resulting in haploid embryogenesis, while fertilization by the other sperm cell creates the triploid endosperm (Geiger 2009).

In nature, haploid frequency is low in maize. In Chase's (1949) study, the average haploid frequency was 0.1% (Coe 1959). However, there are unique genotypes that have the ability to induce a higher than normal frequency of haploid seedlings (Chang and Coe 2009). One such genotype is RWSxRWK-76, developed at the University of Hohenheim, which has an average induction rate of 9–10% (Geiger 2009). RWS and RWK-76 are related lines, but their F_1 is more vigorous and sheds more pollen compared to either parental line (Geiger 2009). Like most inducers, RWSxRWK-76 is equipped with the *R1-nj* (Navajo) marker gene to distinguish haploid from hybrid or contaminated kernels. Any hybrid kernel will display a purple cap on the endosperm and a purple scutellum if anthocyanin marker expression is not inhibited by colour inhibitor genes (Chang and Coe 2009, Geiger 2009). Haploid kernels have a purple cap on the endosperm, but the scutellum is colourless, and contaminants will have no colour markings at all (Chang and Coe 2009, Geiger 2009).

Haploid inducers equipped with other phenotypic markers like transgenic herbicide resistance, high oil trait or red root marker can be used. With use of a transgenic herbicide-resistant inducer, seedlings are treated with herbicide on a small area of a leaf; leaf tissue injury results in haploid seedlings (Geiger 2009). The high oil inducer line produces diploid seed with larger embryo size and higher oil content than haploid seed and progeny can be identified using nuclear magnetic resonance (Chang and Coe 2009). Rotarenco et al. (2010) described use of a red root marker to discriminate haploid from diploid progeny of induction crosses. Investigations by Chaikam and Prasanna (2012), Chaikam et al. (2016) have established the usefulness of the red root anthocyanin marker, especially when *R1-nj* expression is inhibited or expression segregates. *R1-nj* expression can be an issue with identification of haploid progeny from germplasm derived from tropical lines or landraces, and certain other backgrounds.

Genome doubling in haploid individuals is challenging. The range of spontaneous fertility restoration of the ear ranges from 25% to 94% (Chalyk 1994, Liu and Song 2000, Han et al. 2006, Chang and Coe 2009), and restoration of tassel fertility ranges from 2.8% to 46% (Shatskaya et al. 1994, Liu and Song 2000, Han et al. 2006, Wei and Chen 2006, Chang and Coe 2009). The low percentage of tassel fertility restoration is the major limiting factor to producing seed from a haploid plant. In addition to the limitations for spontaneous fertility restoration, the haploid plant is less vigorous and more likely to show symptoms in a stressful environment (Chase 1952, Chalyk 1994, Geiger 2009). Haploid plants treated with colchicine in the seedling stage to effect chromosome doubling are chimeric, as genome doubling occurs in some but not all cells of the plant (Chang and Coe 2009, Geiger 2009). Genome doubling of a cell with a haploid genome can be the result of somatic cell fusion (of two haploid cells), endoreduplication, endomitosis or possibly other mechanisms (Jensen 1974, Testillano et al. 2004, Chang and Coe 2009). The lower a cell's DNA content, the more likely the cell will undergo endopolyploidization (Barow 2006).

Genome doubling rates can be increased by proper treatments. Kato (2002) developed a method to treat haploid plants with

nitrous oxide gas. However, this method is very tedious and specialized equipment is required (Chang and Coe 2009, Geiger 2009). Another option is the use of a colchicine treatment. One of the methods used by Eder and Chalyk (2002) treats haploid seedlings at the three- to four-leaf stage with a 0.125% colchicine solution containing 0.5% dimethyl sulphoxide injected right above the growing point in the stem. Use of artificial doubling methods has resulted in an average DR of 44% with nitrous oxide (Kato 2002) or 27.3% for the colchicine injection method used by Eder and Chalyk (2002), higher than the 3.3% found in spontaneous doubling haploids by Chalyk (1994).

Until recently, the use of DH technology in maize has not really been utilized or evaluated for tropical germplasm (Belicuas et al. 2007). Tropical inducer candidates that could produce a ten per cent induction rate in a tropical environment were recently reported (Prigge et al. 2012). GEM breeding crosses of tropical x temperate germplasm were evaluated based on various breeding methods including the DH technology, and it showed that the DH method was as effective as other breeding methods (Jumbo et al. 2011). The GEM programme provides temperate-adapted tropical sources that are useful in evaluating tropical materials in a temperate environment. It is a public and private collaboration to increase and improve the diversity of elite US maize germplasm (Pollak 2003). Using the traditional GEM protocol, germplasm from 20 countries has been adapted to the Corn Belt of the USA. A new GEM effort, 'allelic diversity' (AD), was established with the objective of sampling previously unused races and developing a comprehensive collection of adapted maize lines with introgressions of exotic alleles from ~300 exotic races of maize, including representation of rare alleles. These exotic donor races are too late and photoperiod sensitive to be used for crosses in the Midwest directly. Therefore, each race is crossed to two expired plant variety protection (PVP) lines, PHB47 (stiff stalk heterotic group) or PHZ51 (non-stiff stalk group), after photoperiod (short day) treatment of the exotic donor populations. Resulting F_1s are usually still unadapted to the Midwest. The F_1s are backcrossed to the respective Ex-PVP lines PHB47 or PHZ51 as the recurrent parent to create backcross 1 (BC_1) families. Most BC_1s are sufficiently adapted to Midwestern conditions to allow line development in Iowa. Using traditional breeding methods of selfing to homozygosity, at least nine seasons are theoretically required to inbreed and increase lines for release, depending on the germplasm involved. Using the DH method, lines could be released as early as four seasons after making the induction cross. Therefore, using the DH method for the AD project would hasten release of germplasm from the 300 races. Concerns with use of *in vivo* DH technology on AD sources are whether (i) choice of elite Ex-PVP line as recurrent parent impacts efficiency of DH line development, (ii) exotic donor parent differences impact HIR and DR, and (iii) origin based on elevation (highland versus lowland) is associated with differences in HIR and DR. In this study, HIR and DR were evaluated in induction crosses involving two elite recurrent parents and 18 donor races from three elevation groups.

Materials and Methods

For the haploid induction study, selected races from the allelic diversity project were assigned to three elevation groups. The high-elevation group consisted of races above 2500 m, mid-elevation races between 1500 and 2499 m, and low-elevation races originating from elevations below 1500 m (Table 1). Six races were randomly selected from each of the three elevation groups for a total of 18 races. These 18 races were crossed to each of two Ex-PVP inbred lines, PHB47 and PHZ51, and backcrossed to the same inbreds, resulting in 36 populations. For the growing seasons of 2011 and 2012, ten replications of each population were planted in a complete randomized block design for haploid induction, providing 360 experimental units each year. Each replication consisted of one row containing at most ten plants. At least three pollinations were attempted using the maternal haploid inducer RWSxRWK-76, to obtain seed for haploid screening. Kernels were sorted during the winters of 2012 and 2013 based on a colour marker to differentiate between hybrids, seed not expressing the *R1-nj* marker and haploids. Any broken, germless or diseased kernels were discarded and not utilized in the calculation of HIR. If the kernel expressed the purple Navajo marker (*R1-nj*) on the cap of the kernel, it was assumed that it resulted from pollination with the haploid inducer and would either be scored as haploid or a hybrid. Kernels without *R1-nj* expression were considered to be 'contaminated' seed due to the inability to identify as a haploid kernel or an outcross. If a kernel had both a purple cap and a purple scutellum, it was considered to be a hybrid and discarded. If a kernel lacked a purple scutellum and had the *R1-nj* marker on the kernel cap, the kernel was considered to be a putative haploid. The number of

Table 1: Maize races evaluated and actual haploid induction rate (HIR)

					Backcrossed to	
Accession no	Race	Country	Altitude (M)	Elevation	PHB47 (%)	PHZ51 (%)
PI 483606	Cacahuacintle	Mexico	2500	High	7.11	5.66
NSL 286967	Chillo	Ecuador	2500	High	7.28	4.57
NSL 285802	Conico	Mexico	2500	High	4.82	3.94
PI 485274	Cuzco	Peru	2850	High	7.37	8.04
PI 503681	Cuzco Cristalino Amarillo	Peru	3250	High	6.28	7.74
Ames 28865	Huancavelicano	Peru	2850	High	6.22	5.97
PI 443776	Amagaceño	Colombia	1750	Medium	4.34	3.93
PI 485359	Arizona	Peru	1500	Medium	5.43	6.26
PI 645786	Celaya	Mexico	1500	Medium	6.20	4.14
Ames 28748	Patillo Grande	Bolivia	2320	Medium	5.49	5.85
PI 444525	Sabanero	Colombia	2400	Medium	5.76	6.73
Ames 28507	San Marceño	Guatemala	2408	Medium	7.06	5.22
PI 444449	Comun	Colombia	1100	Low	7.00	7.85
NSL 286500	Corioco	Bolivia	180	Low	7.31	5.32
PI 489506	Harinoso de Ocho	Mexico	100	Low	7.12	6.44
Ames 28824	Puya Grande	Venezuela	360	Low	6.54	6.06
PI 511649	Tuxpeño	Mexico	250	Low	8.00	4.47
PI 629142	Tuxpeño Norteño	Mexico	500	Low	10.30	6.85

haploid, hybrid and seeds without *R1-nj* expression was recorded to obtain haploid induction rates. The HIR was calculated by dividing the number of haploid seeds by the total number of seeds in the experimental unit. Using a 95% confidence interval, the following model was used to analyse the results:

$$Y_{ijkl} = \mu + B_i + E_j + \mathrm{BE}_{ij} + R_{(j)k} + \mathrm{BR}_{i(j)k}\varepsilon_{ijkl},$$

where Y_{ijkl} represents the number of haploid individuals successfully induced, B = the effect of the ith background, E = the effect of the jth elevation group, BE = the interaction of background and race to the ijth level, R = the effect of the kth race nested in the jth elevation group, BR = the interaction of background and race nested within elevation to the ijkth level, ε = the normal distributed residual error. Using SAS 9.2, probabilities and Tukey–Kramer comparisons between lines using the least squares means were calculated.

The putative haploid seed produced in the induction study was used for the genome doubling study. In spring of 2013 and 2014, ten replicates per population, each with ten putative haploid plants, were planted in pots in a complete randomized design for a total of 360 experimental units per year (720 experimental units in total). All pots were grown in the USDA greenhouses at Iowa State University campus. All putative haploid plants received treatment by injecting a 0.125% colchicine solution with 0.5% dimethyl sulphoxide into the stem above the growing point at the 3- to 4-leaf stage (Eder and Chalyk 2002) to promote doubling. Plants were transplanted to the field after a few days of hardening outside.

During the pollination periods of 2013 and 2014, the DH nursery was examined daily for plants with extruded anthers. If anthers shedding pollen were found, then self-pollination was attempted the same day. If necessary, ear husks were cut back to provide access to silks. If there were multiple shedding anthers, the anthers were tapped onto a bag and pollen released was applied to the available silks. If only one or two anthers were shedding, the anthers would be plucked off the tassel, placed on a pollinating bag, cut open, tapped to release the pollen and the pollen applied to the silks. For statistical data analysis, the following model was used: Y_{ijkl}~Binomial (N_{ijkl}, P_{ijkl}),

where Y_{ijkl} represents the number of individuals with successfully doubled genomes, N_{ijkl} is the final stand of the row, and P_{ijkl} is the probability of doubling for that row, with Ex-PVP background at the ith level, elevation at the jth level, race at the kth level and the row replication at the lth level. Due to assessment of doubling success as a yes/no observation and low number of data points, a binomial distribution was used. DR was calculated by dividing the number of plants bearing an ear with viable kernels (one or more kernels) by the final number of plants per population. Any ears segregating for kernel colour or kernels expressing the Navajo gene were considered to be hybrids and were not included in the calculation.

Results

For haploid induction, across both the 2011 and 2012 growing seasons, populations with low-elevation races had significantly higher HIRs than those with high or mid-elevation groups (P = 0.0003) (Table 2). The low-elevation race populations averaged a HIR of 7.13% (Table 2) and ranged from 6.31% for the Puya Grande race to 8.54% for the Tuxpeño Norteño race (Table 3). The average HIR of the high-elevation race populations averaged 6.24% (Table 2) with a range of 4.47% to 7.62% from Conico and Cuzco races, respectively (Table 3). Middle elevation race populations averaged 5.62% (Table 2) for HIR and had rates that ranged from 4.16% HIR for the Amagaceño race to 6.41% HIR for the San Marceño (Table 3). Recurrent Ex-PVP parent effects were significant (P = 0.0026), with PHB47 having a higher HIR, 6.67%, than PHZ51, 5.90% over the 2 years. When the recurrent parent was PHB47, the HIR ranged from 4.34% for the Amagaceño race to 10.30% for the Tuxpeño Norteño race (Table 1). When PHZ51 was used as the recurrent parent, the HIR ranged from 3.93% to 8.04% for the Amagaceño and Cuzco races, respectively (Table 1). Finally, differences between races were significant as well (P = 0.0001), with Tuxpeño Norteño having the highest and Conico the lowest HIRs when means were adjusted for missing data and analysed using the Tukey–Kramer comparison lines for least squares means (Table 3). HIR for all races averaged 6.37% and ranged from 4.16% to 8.54% for Amagaceño and Tuxpeño Norteño, respectively (Table 3). The highest HIR was 10.30% for Tuxpeño Norteño backcrossed to PHB47, and the lowest HIR was 3.93% from Amagaceño backcrossed to PHZ51 (Table 1). The interaction of background and elevation was non-significant (P = 0.1639); however, the three-way interaction of background and race nested in elevation was significant (P = 0.0066).

For the 2013 and 2014 doubling experiment, the average DR for the experiment was 9.16%. The results were not significant (P = 0.6812) for the elevation groups with the average DR for low elevation at 8.08%, mid-elevation DR at 9.94% and high-elevation DR at 9.69%. The Ex-PVP parent background effect was also not significant (P = 0.6022), with an average DR of 9.11% for races backcrossed to PHZ51 and an average DR of 9.21% for races backcrossed to PHB47. The average DR for races ranged from 4.30% for Tuxpeño Norteño to an average DR of 13.18% for the Sabanero race (Table 4); race DR was not significant (P = 0.9988).

Table 2: Evaluation of maize race origins' elevation on HIR using Tukey–Kramer comparison lines for least squares means

Letter	Elevation	Average HIR (%)	HIR Range[1] (%)	Least squares means HIR (%)
A	Low	7.13	4.47–10.30	6.70
B	High	6.24	3.94–8.04	5.96
B	Middle	5.62	3.93–7.06	5.57

Least squares means with the same letter are not significant.
[1]Actual minimum HIR to maximum HIR.

Table 3: Evaluation of maize race haploid HIR using Tukey–Kramer comparison lines for least squares means

Letter	Race	Average HIR (%)	HIR Range[1] (%)	Least squares means HIR (%)
A	Tuxpeño Norteño	8.54	6.85–10.30	8.22
AB	Cuzco	7.62	7.37–8.04	7.39
AB	Comun	7.36	7.00–7.85	7.15
AB	Harinoso de Ocho	6.85	6.44–7.12	6.71
AB	Cuzco Cristalino Amarillo	6.78	6.28–7.74	6.69
ABC	Sabanero	6.17	5.76–6.73	6.26
ABC	Puya Grande	6.31	6.06–6.54	6.16
ABC	San Marceño	6.41	5.22–7.06	6.15
ABC	Cacahuacintle	6.64	5.66–7.11	6.07
ABC	Tuxpeño	6.60	4.47–8.00	6.07
ABC	Huancavelicano	6.15	5.97–6.22	6.05
ABC	Arizona	5.87	5.43–6.26	5.96
ABC	Corioco	6.49	5.32–7.31	5.91
ABC	Patillo Grande	5.59	5.49–5.85	5.85
BC	Chillo	6.45	4.57–7.28	5.51
BC	Celaya	5.59	4.14–6.20	5.11
C	Amagaceño	4.16	3.93–4.34	4.12
C	Conico	4.47	3.94–4.82	4.01

Least squares means with the same letter are not significant.
[1]Actual minimum HIR to maximum HIR.

Table 4: Actual doubling rates for maize races

Accession no	Race	Country	Altitude (M)	Elevation	DR (%)
PI 444525	Sabanero	Colombia	2400	Medium	13.18
PI 489506	Harinoso de Ocho	Mexico	100	Low	12.29
PI 511649	Tuxpeño	Mexico	250	Low	12.11
NSL 285802	Conico	Mexico	2500	High	11.89
PI 483606	Cacahuacintle	Mexico	2500	High	11.24
NSL 286967	Chillo	Ecuador	2500	High	10.93
PI 485274	Cuzco	Peru	2850	High	10.90
PI 645786	Celaya	Mexico	1500	Medium	10.61
Ames 28507	San Marceño	Guatemala	2408	Medium	10.12
PI 485359	Arizona	Peru	1500	Medium	9.60
Ames 28865	Huancavelicano	Peru	2850	High	8.81
Ames 28748	Patillo Grande	Bolivia	2320	Medium	8.63
PI 444449	Comun	Colombia	1100	Low	7.66
PI 443776	Amagaceño	Colombia	1750	Medium	7.07
PI 503681	Cuzco Cristalino Amarillo	Peru	3250	High	6.11
Ames 28824	Puya Grande	Venezuela	360	Low	5.98
NSL 286500	Corioco	Bolivia	180	Low	5.96
PI 629142	Tuxpeño Norteño	Mexico	500	Low	4.30

Discussion

There were challenges associated with the unique backgrounds of the allelic diversity programme germplasm. *Pr1* or *R1* will create a dark red or purple seed colouring in the aleurone layer (Ford 2000). Expression of these genes for coloured aleurone made it difficult to use the *R1-nj* kernel marker needed for haploid identification. Some of the races likely carry genes that inhibit aleurone colour expression. The Navajo marker will not be expressed if the genotype involved carries any of the three genes (C1-I, C2-Idf and In1-D) that inhibit anthocyanin synthesis (Eder and Chalyk 2002), making discrimination and detection of haploid seeds difficult, and potentially resulting in misclassification during the haploid screening process. These inhibitor genes have been reported to be present mainly in flint backgrounds (Eder and Chalyk 2002, Röber et al. 2005). Several of the races in this study are tropical flints. Perhaps use of a different haploid screening and identification process, that would be unaffected by coloured aleurones or marker suppression, would give different results than those found in our study.

Lowland races have been shown to produce high seed yield independent of the environment, whereas highland races gave good seed set at high elevations only (Mercer et al. 2008). Mid-elevation races performed well independent of the environment, but seed quantities were lower than those of the lowland races (Mercer et al. 2008). In our study, the lower elevation races produced an average of eight per cent more seed (data not shown) than the other two elevations in this experiment for haploid screening, similar to the observations of Mercer et al. (2008). Over all races, approximately 33% more putative haploid and total seed was produced, when the recurrent parent was PHB47 compared to PHZ51 (data not shown). As PHB47 is a stiff stalk female, and commercial females are selected for high seed parent yield, this is not surprising. More seed increases the odds of finding more accurately identified putative haploid seeds, which could explain the significant Ex-PVP recurrent parent effect on HIR. Also, significantly more seed without the *R1-nj* expression was found when PHZ51 was the recurrent parent ($P = 0.0001$), which probably contributed to the lower HIR, as this category probably harbours putative haploids lacking readily detected visual marker expression.

With the PHB47 recurrent parent having a higher HIR than PHZ51 in the induction study, it would be interesting to evaluate whether or not stiff stalk lines are prone to producing proportionally more haploids than non-stiff stalk lines. This study could identify potential PVP lines that would better serve as recurrent parents, in order to facilitate the process of developing DH lines from GEM's AD project. A recent publication found that HIR of tropical germplasm being induced for haploids can vary significantly (Prigge et al. 2012). The HIR of tropical x temperate crosses used in our study also varied significantly. The observed higher HIR in this study using recurrent parent PHB47 implies it would be beneficial for a plant breeder to select breeding populations with high seed production backgrounds if the goal is to efficiently use the DH breeding method, thus optimizing the potential to derive more DH lines for selection. This will help ensure that sufficient haploid seeds can be detected for use in the doubling phase of this breeding method.

Although there were enough experimental units to evaluate all the questions raised in the haploid doubling study, growing season conditions affect doubling. In our previous DH breeding experience, hot and dry years seem to reduce the doubling rate, while cooler wetter seasons seem to increase observed doubling rates. The summer of 2013 was cooler, but also drier than normal when this experiment was conducted. The 2014 growing season was cooler and wetter and the overall DR did increase from 8.34% in 2013 to 10.26% in 2014. There were 463 experimental units (out of 720) that yielded zero doubled haploids, which impacts the ability to detect differences in the study. It would have been advantageous to have fewer experimental units and longer rows (more plants per unit) to help detect differences. This would have increased the chance of observing some rate of doubling in each row, reducing the amount of experimental units yielding no doubled haploids. Previous doubling outcomes observed in 2009 were used to run a simulation to determine this experiment's design and number of experimental units needed to detect differences. However, that year's (2009) data seem to have been an exception when producing DH lines due to the higher DR when compared to this study's outcome. It may be worth re-running the simulation using this study's results to determine the number of experimental units needed to detect significant differences. Conducting this experiment under more

controlled environments such as a greenhouse might be helpful, but would limit the number of experimental units that could be studied.

In doubling haploid germplasm, it has been shown that doubling rates of tropical landraces lag behind those of more elite tropical germplasm or Corn Belt materials (Kleiber et al. 2012). This is consistent with the low doubling rates observed in this experiment. It has also been found that some genetic backgrounds will respond to use of colchicine for artificial doubling better than others (Ragot and Steen 1992). Perhaps the Ex-PVP backgrounds and landraces used in this study are less amendable to the use of colchicine for artificial doubling than other germplasm sources. Results of this study suggest that increasing the rate of doubling from artificial induction methods is more likely to be successful than trying to identify genetic backgrounds in tropical landraces that are more conducive to doubling, regardless of their elevation, origin or Ex-PVP background used for temperate adaptation.

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