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Weighing in on a method to discriminate maize haploid from hybrid seed

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With 2 tables

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Abstract

The doubled haploid breeding method can produce maize inbred lines faster than traditional methods, but there are challenges associated with it. Sorting haploid from hybrid seed based on visual colour markers is time consuming and can be difficult due to colour inhibitors that obscure pigmentation needed to distinguish between haploid, hybrid and out-crossed seed. In this study, weight was evaluated as a method to sort haploid from hybrid seed. A first experiment utilized two families for analysis in a preliminary study. Eleven haploid and hybrid kernels from both families were weighed for a total of 44 experimental units. A second experiment was carried out using six families, using the same format as the previous, for 132 experimental units. Hybrid seed weighed significantly more than haploid seed in both experiments. However, the interaction between line and kernel type was significant in the second experiment. In conclusion, efficacy of sorting haploid from hybrid kernels based on weight depends on the genotypes involved.

Key words: doubled haploid — sorting kernels — maize breeding

Producing inbred lines in maize can take up to eight seasons, when using a conventional breeding protocol, but as few as three seasons with the use of the doubled haploid (DH) breeding method. The first step to create DH lines is to induce haploid individuals. Two options are available to achieve this goal. The first option is to utilize *in vitro* techniques, such as anther culture to produce haploids. *In vitro* methods are time consuming, meticulous and have been inefficient in producing haploid individuals in maize (Chang and Coe 2009, Geiger 2009). The second option is to use *in vivo* methods, which utilize a haploid inducer genotype to promote haploid progeny in a cross-pollination. Male inducer lines utilize the Navajo (R1-nj) marker to distinguish between outcrossed, haploid and hybrid seed progeny. A cross made using a haploid-inducing genotype will produce a limited percentage of haploid individuals. RWSxRWK-76 is a maternal haploid inducer, developed at the University of Hohenheim and has a haploid induction rate of about 10% (Geiger 2009). Individual haploid and hybrid seed from a successful cross made with RWSxRWK-76 (male) will display a purple cap on the top of the kernel. Kernels not displaying the purple cap are considered to be an outcross and are discarded. Hybrid kernels will display a purple scutellum on the embryo of the kernel and can be discarded. Haploid kernels do not have a purple scutellum, distinguishing them from hybrid seed. To identify putative haploid kernels based on colour markers is a time-consuming effort, and not all maize backgrounds will display good visual markers. There are three colour inhibitor genes (C1-I, C2-Idf and In1-D) that disrupt anthocyanin pigmentation (Eder and Chalyk 2002) and are typically found in flinty endosperm backgrounds (Eder and Chalyk 2002, Röber et al. 2005).

There are other methods for distinguishing haploid individuals, such as the use of a transgenic herbicide-resistant inducer and testing the progeny to identify herbicide susceptible, haploid individuals (Geiger 2009). Another method uses a high oil inducer genotype and uses oil concentration to differentiate between haploid and hybrid seed (Chang and Coe 2009, Geiger and Gordillo 2010). This method produces haploid embryos that are smaller than the hybrid embryos resulting from that cross (Chang and Coe 2009). If this is also true for high oil inducers, could haploid embryos be smaller than other embryos from crosses with any inducer line that employs the R1-nj colour marker to distinguish between haploid, hybrid and out-crossed seed? If so, would haploid seed weigh significantly less than the hybrid seed from that cross, and could it serve as a factor for distinguishing haploids with genes that inhibit R1-nj expression? In this study, seed weight was evaluated for use as a metric to differentiate between haploid and hybrid progeny among publicly available lines, cross-pollinated with the RWSxRWK-76 inducer.

Materials and Methods

In the winter of 2011, 5 haploid seeds and 5 hybrid seeds of B73 were sent to Strube Research GmbH & Co. KG, in Germany for evaluation on various seed characteristics. Using a simple ANOVA, weight was identified as being significant (data not shown) for kernel type (haploid or hybrid). From this point, the Agriculture Experiment Station Consulting Group from the Department of Statistics at Iowa State University for statistical support ran simulations from the data set of the B73 trial and determined that 22 haploids and 22 hybrids were needed for evaluation to test weight as a method to differentiate kernel types with 90% confidence on the outcome.

In the summer of 2011, A632, G80, Mo17 and LH82 were pollinated using RWSxRWK-76 to produce haploid seed, and PHB47 and PHZ51 were pollinated in the summer of 2012 with the same haploid inducer. G80, LH82, PHB47 and PHZ51 are expired Plant Variety Protection (Ex-PVP) lines, and A632 and Mo17 are popular public inbred lines. A632, PHB47 and G80 all belong to the stiff stalk heterotic group and Mo17, LH82 and PHZ51 belong to the non-stiff stalk heterotic group.

In the winter of 2011/2012, the haploid and hybrid progeny from the A632 and Mo17 crosses were selected and screened for seed size using 0.31 cm and 0.15 cm diameter screens (Experiment 1). Screening was used to create a pool of haploids and hybrids that would be of similar dimensions for the study and to take year affect out of the equation. Eleven putative haploid seeds and eleven hybrid seeds, as determined by

R1-nj expression, were selected at random for each line from the screened pools of seed (44 experimental units). The selected haploid and hybrid seeds of these two lines were sent to Strube Research GmbH & Co. KG, Soellingen, Germany, for preliminary evaluation of weight as a method for distinguishing haploid from hybrid seed. Strube Research GmbH & Co. KG, Germany, used a Mettler Toledo scale with a sensitivity of 0.1 mg to weigh each kernel. An ANOVA was run using the following statistical model, with LS means calculated, and a 90% confidence interval used:

$$Y_{ijk} = \mu + L_i + T_j + LT_{ij} + \varepsilon_{ijk}$$

Y = the response observed for the ijk^{th} experimental unit, L = the line at the i^{th} level, T = kernel type at the j^{th} level, LT = the interaction between the line and kernel type at the ij^{th} level, ε = the standard error at the ijk^{th} level. L and T are fixed effects, and a normal distribution was used. Data were analysed using the below code for SAS 9.2.

```
proc glm;
class pedigree type;
model weight = pedigree|type;
lsmeans pedigree|type/lines pdiff
```

In the winter of 2012/13, 11 haploid and 11 hybrid seeds of all six lines (132 experimental units) were screened for seed size using 0.31 cm and 0.15 cm diameter screens and weighed using an Ohaus scale that measures with a resolution of 0.1 mg at the North Central Regional Plant Introduction Station in Ames, Iowa (Experiment 2). Haploid and hybrid A632 and Mo17 kernels from the cross with the inducer genotype were reselected, at random, and measured, to confirm the accuracy of the scale and results of the second experiment. This experiment was analysed using the same model mentioned above, but with a 95% confidence level due to use of more experimental units than the prior experiment.

Results

In Experiment 1, the average kernel weight among lines was non-significant at a 10% level (Pr = 0.5150), and kernel type was significant (Pr = 0.0007), with hybrid seed weighing significantly more than haploid seed. The average kernel weight of haploid kernels was 273.2 mg and the average hybrid kernel weight was 316.4 mg. The interaction of the line and kernel type was non-significant (Pr = 0.1396). However, A632 was the only genotype where kernel types were significantly different from each other (Table 1). A632 haploid kernels averaged 268.2 mg and A632 hybrid kernels averaged 329.1 mg for weight, whereas Mo17 haploids weighed 278.2 mg and Mo17 hybrids weighed 303.6 mg on average (Table 1).

In Experiment 2, genotype was significant at a 5% level (Pr = 0.0001) as well as the interaction of line and kernel type (Pr = 0.0119). Kernel type remained significant (Pr = 0.0003) with haploid kernels weighing on average 305.1 mg, while hybrid kernels weighed 328.3 mg on average. When evaluating kernel type within lines, haploids kernels weighed less than hybrid kernels except for G80. The average G80 haploid kernel weight was 362.3 mg, and the average G80 hybrid kernel weight was 342.6 mg (Table 2). G80 has large seed size and has been utilized in our DH research to estimate induction frequency due to the easily distinguishable marker in the embryo to differentiate hybrid seed from putative haploids. A632 and PHZ51 were the only lines with significantly different haploid and hybrid kernel weights (Table 2).

Table 1: Experiment 1: Comparison of LS means for interaction of the line and kernel type

Letter	Line	Accession #	Kernel type	Average weight
A	A632	PI 587140	Hybrid	329.1 mg
AB	Mo17	PI 558532	Hybrid	303.6 mg
BC	Mo17	PI 558532	Haploid	278.2 mg
C	A632	PI 587140	Haploid	268.2 mg

Same letter are NOT significantly different.

Table 2: Experiment 2: Comparison of LS means for interaction of the line and kernel type

Letter	Line	Accession #	Kernel type	Average weight
A	G80	PI 601037	Haploid	362.3 mg
A	PHZ51	PI 601322	Hybrid	357.0 mg
A	A632	PI 587140	Hybrid	348.8 mg
AB	G80	PI 601037	Hybrid	342.6 mg
BC	PHB47	PI 601009	Hybrid	314.6 mg
CD	LH82	PI 601170	Hybrid	306.4 mg
CD	PHZ51	PI 601322	Haploid	305.7 mg
CD	Mo17	PI 558532	Hybrid	300.3 mg
CD	A632	PI 587140	Haploid	297.8 mg
CD	PHB47	PI 601009	Haploid	292.9 mg
CD	Mo17	PI 558532	Haploid	288.2 mg
D	LH82	PI 601170	Haploid	283.7 mg

Same letter are NOT significantly different.

Discussion

These experiments verified that haploid kernels weighed significantly less than hybrids in both experiments, and that kernel weight could be utilized to enrich selection for haploid kernels. It was not surprising that the type of line and interaction between the line and kernel type was significant in the second experiment due to the larger number of lines and experimental units evaluated. As the interaction between line and kernel type was significant in experiment two, the efficacy of successfully identifying haploids in multiple backgrounds may not be sufficient for some breeding programmes due to the need for a stringent threshold to avoid false positives. We evaluated whether heterotic grouping impacts haploid – hybrid seed weight differentiation. In Experiment 2, three stiff stalks (G80, PHB47 and A632) and three non-stiff stalk lines (LH82, PHZ51 and Mo17) were evaluated for haploid and hybrid weight, and only A632 and PHZ51 showed significant differences among haploid and hybrid kernels for weight. For the lines tested, there was no significant impact of heterotic pool identity on haploid – hybrid seed differences (data not shown). As G80 exhibited heavier haploid than hybrid seed, it is advisable to evaluate a small sample of a population prior to running a complete sample through an automated sorting process. A larger sample size might have created a different outcome in the weight distribution found in the G80 population, and it would have increased the power of the experiment to detect significant differences. True flinty backgrounds were not addressed in this experiment due to haploid and hybrid seed being unavailable during the time in which the study was carried out. Screening seed prior to haploid sorting in order to produce similarly sized samples was important to the analysis and outcomes of this study. The next step in using weight as a tool for haploid and hybrid differentiation would be to conduct an experiment where various heterotic backgrounds are induced and putative haploid kernels sorted based on weight alone and by colour marker alone, grow them out, evaluate and compare the rates of false positives (hybrids) found within the putative haploid groups.

Using weight to discriminate haploid from hybrid seed would likely be less efficient for enrichment of haploids compared to colour markers or using a high oil inducer. Utilizing a high oil inducer would also be a more accurate, high-throughput method than using the weight of the kernels. One study found that haploid identification using a high oil inducer can be 90% accurate (Chen and Song 2003, Chang and Coe 2009). However, if a high oil inducer line is unavailable, then differentiating haploid and hybrid seed based on weight will be much faster than the use of colour markers alone. New methods could be developed evaluating three dimensional (3D) computed tomography (CT) scanning technology. 3D CT uses a sealed microfocal X-ray tube that utilizes a manipulation system, a flat panel x-ray detector and a system of computers for acquiring the data, and reconstruction and processing of the 3D image. This technology is currently used by Strube Research GmbH & Co. KG on sugar beet seeds to evaluate the morphological and anatomical features of the seed that correlate to the seedling emergence capabilities. The use of the 3D CT scanning technology could be used as a non-destructive, high throughput way to observe differences in the seed between haploids and hybrids.

Currently, visual sorting is the most common method for distinguishing haploid seed from hybrid seed. Data reported in this study suggest that moving to an automated system that uses weight evaluation to differentiate haploid individuals from hybrid is an option to consider. One could set a very stringent limit on weight that would result in loss of haploid individuals in the higher weight category. This would reduce field effort and resources needed for roguing hybrids, but more induction crosses would be necessary to obtain sufficient haploid seeds to satisfy a required number of doubled haploid individuals, postweight screening process. One could also set a very loose limit on weight, resulting in an increase of hybrid individuals found in the field after sorting. This would increase hybrid roguing efforts, but would also increase the number of haploids making it through the screening process and reduce the number of induction crosses needed. Using either method, there would be a dramatic decrease in the amount of time and labour required to separate haploid from hybrid seed compared to visual identification. Judgement will be required to determine the balance between excluding haploid individuals in the upper weight distribution versus including more hybrid individuals that will be mixed in with the heavier haploids.

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