Core Ideas

- There are many potential benefits of incorporating embryo culture into a doubled haploid program.
- There is no known non-transgenic method of selecting haploid embryos following embryo culture.
- Our goal was to establish a non-transgenic haploid selection method following embryo culture.
- These methods allow early haploid selection based on differential root growth features.

Utilization of Reduced Haploid Vigor for Phenomic Discrimination of Haploid and Diploid Maize Seedlings

Kimberly Vanous, Talukder Zaki Jubery, Ursula K. Frei, Baskar Ganapathysubramanian,* and Thomas Lübberstedt*

Potential benefits of incorporating embryo culture (EC) into a doubled haploid (DH) program, including shortening the breeding cycle and increasing chromosome doubling rates, make the laborious and tedious task of excising embryos worth the effort. Difficulties arise during embryo selection considering the marker gene R1-nj, which is typically used in DH programs, is not expressed in early stages after pollination. Although transgenic approaches have been implemented to bypass this issue, there is so far no known non-transgenic method of selecting haploid embryos. The findings of this study reveal methods of selecting haploid embryos that allow the possibility of incorporating EC into a DH program without using transgenic inducers. The best performing method involves a machine-learning classifier, specifically a support vector machine, which uses primary root lengths and daily growth rates as traits for classification. Selection by this method can be achieved on the third day after germination. By this method, an average false negative rate of 2% and false positive rate of 9% was achieved. Therefore, the methods presented in this research allow efficient and non-transgenic selection of haploid embryos that is simple and effective.

Embryo culture (EC) is an in vitro technique for germination of immature embryos. The most common application is rescue of interspecific and intergeneric diploids that would otherwise abort due to an improperly developed endosperm (Bridgen, 1994). An artificial medium provides nutrients that the endosperm would otherwise supply. Therefore, this technique is also called “embryo rescue.” Because EC is one of the oldest and most perfected in vitro procedures, a broad range of uses have developed over time. Embryo culture can be utilized to shorten the generation cycle by bypassing seed dormancy. In maize (Zea mays L.) doubled haploid (DH) programs, the generation cycle is shortened by approximately 6 wk if the embryo is excised 10 to 12 d after pollination (DAP). In addition, EC can be used in DH programs to increase genome doubling rates by adding colchicine to the growth medium. Embryo culture was shown to increase the effectiveness of colchicine treatment from 10 to >90%, which is a dramatic increase in efficiency of DH line production (Barton et al., 2014).

Most DH lines in maize are produced by maternal haploid induction—in vivo production of haploids by pollinating a donor plant with inducer pollen. Maternal in vivo haploid induction results, on average, in 8 to 12% haploid progeny in maize, probably by fertilization and subsequent chromosome elimination (Liu et al., 2016; Ren et al., 2017). Since the inducer genome carrying the dominant anthocyanin marker gene R1-nj (Navajo) (Nanda and Chase, 1966; Neuffer et al., 1997) is eliminated, haploid kernels can be visually selected based on this marker. Visual selection is done after harvest based on mature kernels. Diploids will be identifiable because both the endosperm (3n) and embryo (2n) will be red or purple colored, whereas haploids have a red or purple colored endosperm (3n) but a colorless embryo (n). Once haploids are selected, they are typically treated with a mitotic inhibitor, such as colchicine, to restore diploidy and fertility

Abbreviations: AUC, area under curve; DAP, days after pollination; DH, doubled haploid; EC, embryo culture; FNR, false negative rate; FPR, false positive rate; GR, growth rate; PETG, polyethylene terephthalate glycol; PRL, primary root length; SVM, support vector machine.

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Received 20 Oct. 2018.
Accepted 18 Feb. 2019.
*Corresponding author (baskarg@iastate.edu; thomasl@iastate.edu).

in a chimeric pattern (Vanous et al., 2017). Successfully doubled haploids are self-pollinated to create DH lines.

Unfortunately, the most commonly used marker for DH production, R1-nj, is not practical to use for EC. Expression levels correlate with kernel maturation (Alexander and Cross, 1983), and an embryo harvested 10 to 12 DAP will not express R1-nj. Earlier expression of the marker can be achieved by replacing the native promoter with the aleosin or Lectin D (Lecd) promoters, which are expressed as early as a few hours after pollination (Barton et al., 2014). However, regulatory constraints in using genetic modifications may limit the use of transgenic inducers. Generally, DH lines do not contain inducer genes because the inducer chromosomes are eliminated. However, this is only an assumption because the biological process of haploid induction is still not fully understood. There are two hypotheses on the genesis of haploids: single fertilization (i.e., failure of fusion between sperm and egg) and postzygotic genome elimination (i.e., inducer chromosomes are eliminated after double fertilization) (Sarkar and Coe, 1966; Zhao et al., 2013). Observations of haploids carrying inducer chromosome segments support the hypothesis of postzygotic genome elimination. When CAU5 was used to produce maternal haploids, about 43% of the haploids carried inducer chromosome segments (Li et al., 2009). In addition, when a CAU5B inducer carrying a B chromosome was crossed with a diploid, a low frequency of haploids were found carrying B chromosomes and a haploid plant carried a 44 Mb inducer segment (Zhao et al., 2013). Thus, DH lines produced by transgenic inducers may be regulated under genetically modified organism (GMO) legislation (Murovec and Bohanec, 2012).

The overall goal of this study was to establish an efficient non-transgenic method for early selection of haploid embryos following EC. It is well established that haploids show reduced vigor compared with diploid plants (Chase, 1964). We investigated methods of early haploid selection based on differential root growth features of haploid and diploid plants during EC. Our specific objectives were to: (i) explore biological differences of root characteristics between embryos of haploid and diploid genotypes; (ii) develop a statistical method to reliably discriminate between haploid and diploid embryos within 1 wk of EC; and (iii) design a simple and streamlined selection protocol.

### Materials and Methods

#### Plant Materials

Both haploid and diploid embryos were produced by crossing RWS/RWK-76 (Röber et al., 2005) with two inbred genotypes, Wf9 (Ames 19293; Abel et al., 1995; Flint-Garcia et al., 2005) and PHZ51 (PI 601322), in Ames, IA, during the summer of 2015. Four pollination dates were staggered about 1 wk apart and considered as four independent experiments. Five to six pollinations were made at each date to account for poor pollination or poor seed set. The four best ears for each inbred were harvested after 539 to 609 growing degree days, which equated to 28 to 33 DAP. Although the harvest date is preferred to be 10 to 12 DAP to shorten the generation time as much as possible, the harvest date for this study was delayed to allow selection based on the R1-nj marker. This selection was necessary to ensure isolation of a sufficient number of haploids, given their low frequency. Eight embryos of both haploids and diploids were isolated from each ear for each independent experiment. This resulted in a total of 256 embryos isolated and analyzed for each inbred.

#### Embryo Culture Procedure

Embryos were directly disinfected by submerging them in 10% (v/v) commercial bleach for several seconds, followed by a washing step in sterile, distilled water for several minutes before placement on growth media. The growth medium used was a 6% gel at a 3:1 phytagel/agar ratio, and the main components included full-strength Murashige and Skoog (1962) salts, 2% sucrose, and thiamine HCl (0.2 mg/L), and thiamine HCl (0.2 mg/L) at a pH between 5.7 and 5.8.

The containers used in this study were prepared by sterilizing them in 30% (v/v) commercial bleach for 15 min, followed by two washes in sterile distilled water for 5 min each. The containers were dried completely for several hours in a fume hood under sterile conditions. After they were dry, MS gel medium was poured into the plate for media. Strips were attached vertically with double-sided tape within this basin to separate each well and further define the growing space for each embryo. Once medium was poured and solidified, the plate was set vertically upright inside a growth chamber (Fig. 1). These plates are cheap (only US$2 per plate) and reusable after soaking in a 30% (v/v) bleach solution for sterilization.

#### Designing and Building Custom Plates

To acquire repetitive two-dimensional images of roots during EC over the course of development, a specialized container was custom designed and built for this study. Materials to build the containers included polyethylene terephthalate glycol (PETG) plastic sheets (0.3 m by 0.4 m by 4 mm from McMaster-CARR), polyvinyl chloride foam double-sided tape with acrylic adhesive (1.6 mm thickness from McMaster-CARR), and binder clips (12.7 mm), which were inexpensive, simple to acquire, and reusable. An essential characteristic of these containers was that they were very thin (3.2 mm) to discourage roots from overlapping or growing in a three-dimensional space.

To build the plates, several PETG plastic sheets were cut into 6-mm-wide strips. These strips were attached to three out of the four edges of a full plastic sheet using double-sided tape. A flexible sealant, such as putty or dough, was used along the fourth edge of the plate to create a basin to pour media. Strips were attached vertically with double-sided tape within this basin to separate each well and further define the growing space for each embryo. Once medium was poured and solidified, the flexible sealant was removed, embryos were arranged in each well, another PETG plastic sheet was clamped to the plate with binder clips, and the plate was set vertically upright inside a growth chamber (Fig. 1). These plates are cheap (only US$2 per plate) and reusable after soaking in a 30% (v/v) bleach solution for sterilization.
dark environment at 25°C. Each seedling was photographed every 24 h for up to 12 d. When the containers were no longer needed for imaging, they were disassembled, sanitized, and reused for the next experiment. DNA was extracted from each seedling before disposal. Public SSR marker p-umc2390, located in chromosomal bin 1.04, was used to confirm that the samples were truly haploid or diploid.

Data Acquisition and Extraction of Image-Derived Primary Root Length Measurements

Each container, with four seedlings each, was photographed every 24 h for up to 12 d. The camera used was a Panasonic Lumix DMC-TZ5 with a resolution of 460,000 total pixels. In each imaging session, both camera and plate rack were placed in the same position. A shadowbox placed between the camera and the container minimized reflections since the images were taken through the PETG. A fixed setup of the imaging platform allowed consistent imaging, as only one image needed to include a ruler for scaling purposes. ImageJ (Schneider et al., 2012) was used to extract the primary root length (PRL) for each root by measuring the number of pixels, which was later converted to centimeters based on the scaling object.

Analyses were performed with PRL measurements (i) based on the period between the start of EC and variable end dates (Method A), (ii) for the period between the first day of visible root growth and variable end dates to adjust for germination differences (Method B), (iii) for growth rate (GR) based on data from Method B during the first 9 d (Method C), and (iv) by support vector machine (SVM) learning (Method D). Because some embryos did not germinate until after 3 to 4 d, time periods considered for Method B were maximally 9 d long. The GR was measured by taking PRL measurements and fitting a linear regression for these data points. The slope of the regression line of each sample was used in the statistical analysis.

Statistical Analyses and Selection Method Analyses

The statistical software package SAS 9.4 (SAS Institute) and the function PROC MIXED was used to analyze PRL data by days after germination and by GR as well as germination date. The following model was used for variance analysis:

$$y_{ijk} = \mu + E_i + G_j + (EG)_j + P_k + (EP)_jk + (GP)_jk + E_{ijk}$$

where $y_{ijk}$ represents the observation of the $ijk$th experimental unit. Main effects are represented by $E_i$ or the $i$th experiment, $G_j$ or the $j$th genotype, and $P_k$ or the $k$th ploidy level. The error term is defined as $E_{ijk}$. The function PROC MIXED was used for analysis of Type 3 sums of squares. The function PROC CORR was used to separately compare haploid and diploid PRL measurements among all days after germination.

Receiver operating characteristic curves were derived using PROC LOGISTIC and were used for the analysis of selection method performance. The method with the highest area under the curve (AUC) was designated as the preferred selection method (DeLong et al., 1988). Output results were used to compute Youden’s $J$ statistic:

$$J = \frac{TP}{TP + FN} + \frac{TN}{TN + FP} - 1$$

Fig. 1. Steps in embryo culture EC using the custom-designed polyethylene terephthalate glycol (PETG) plates: (a) a flexible sealant or alternative barrier along the top edge (b) creates an enclosed basin to pour medium; (c) once the medium has solidified, the barrier can be removed and the plate is ready for (d) embryo isolation and (e) placement; (f) PETG sheeting is used to cover the setup and (g) clamped into place so it can be set vertically for (h) root growth.
where TP and TN indicate true positives and true negatives, respectively, while FP and FN indicate false positives and false negatives, respectively. The \( J \) statistic ranges from 0 to 1, with 0 being a non-informative and 1 a perfect test. Youden’s index was used to derive the optimal threshold for selection (maximum \( J \) statistic). Samples with values below this threshold are predicted to be haploids. Youden’s index is based on equal weights to FP and FN results, and it does not consider the prevalence of haploids (\( P \)). Current haploid inducer lines have about 10% induction rates (Liu et al., 2016), future inducers are likely to have induction rates above 20%. Therefore, two scenarios were computed where \( P = 10\% \) and \( P = 20\% \), and in both cases a heavier weight was placed on FN results, thereby capturing as many haploids as possible without overly compromising precision. For these scenarios, optimum criterion thresholds were determined by finding the point of intersection between the receiver operating characteristic curve and a line in which the slope is calculated by considering the ratio of the costs of false results as well as the prevalence of haploids (Zweig and Campbell, 1993). The slope of this line is calculated as

\[
m = \frac{F_{PC} \times (1 - P)}{F_{NC} \times P}
\]

where \( m \) is the slope of the threshold-determining line, \( F_{PC} \) and \( F_{NC} \) denote the costs of false positives and false negatives, respectively, and \( P \) represents haploid prevalence. In both cases (\( P = 10 \) or 20\%), the ratio of negative results (\( F_{PC}/F_{NC} \)) was 1:5, placing more weight on false negative results.

Sensitivity and specificity are properties of the selection method and were used in determining false positive rates (FPR) and false negative rates (FNR) when considering application of the optimal criterion threshold determined by Youden’s \( J \) statistic. For the application of optimal criterion thresholds in both scenarios, the FPR\(_P\) and FNR\(_P\) were calculated as

\[
FPR_P = (1 - SPC)(1 - P) = FPR(1 - P)
\]

\[
FNR_P = (1 - SNS)P = FNR \times P
\]

where FPR\(_P\) and FNR\(_P\) are false positive and false negative rates given a particular haploid prevalence (\( P \)), and SPC and SNS refer to specificity and sensitivity, respectively, for the selection method of choice.

**Machine Learning Approach**

Analysis by SVM learning-based classification involved a training and a validation step. For the training step, 75% of the haploids and 75% of the diploids (determined by \( R1-nj \) selection and DNA analysis) were randomly selected from the dataset and used to develop the best classifier. A function that is a linear combination of the root traits was developed for a classifier, allowing the maximal margin separating haploids from diploids. Specifically, traits were the PRL for a given day (e.g., PRL for Day 3), all PRLs for days prior to a given day (e.g., PRLs for Days 1–2), and daily growth rates (e.g., daily growth rate = PRL for Day 3 − PRL for Day 2).

The SVM-based classification used in this research utilized a radial basis function classifier. Radial basis function is a kernel function that transformed the data (root traits) and allowed linear separation between groups of interest. The equation for the radial basis function is

\[
f(x_i) = \exp\left(-\gamma \|x_i - x_j\|^2\right)
\]

where \( x_i \) and \( x_j \) are vectors that contain traits related to the support vectors and the normalized root traits of interest, respectively, and \( \gamma \) is the kernel scale parameter. A Bayesian optimization strategy was deployed to identify the optimal scale parameter.

Samples were then classified for ploidy by inserting the above \( f(x_j) \) expression into a separate function, resulting in the classifying output (\( Y \)). Geometrically, the function represents a hyperplane that separates haploids and diploids. Negative outcomes would result in a classification of haploid, whereas positive outcomes would result in a classification of diploid, with more negative values indicating a higher likelihood that samples are truly haploid. The function to produce classifying output values is

\[
Y = w_0 + \sum w_i f(x_i)
\]

where \( w_0 \) is the bias term and \( w_i \) are the weights related to the support vectors. In the validation step, classifiers were used to identify haploids in the remaining 25% of the data.

**Results**

Two inbred lines, Wi9 (Ames 19293; Abel et al., 1995; Flint-Garcia et al., 2005) and PHZ51 (PI 601322), were induced by crossing them with haploid inducer RWS/RWK-76 (Röber et al., 2005) to produce embryos segregating for ploidy. Both haploid and diploid embryos were harvested from this material for EC. Embryos were grown in specialized containers, which allowed continuous monitoring of root development. Primary root length (PRL) was measured for each sample every 24 h for the first 2 wk of growth.

**Primary Root Development in Embryo Culture**

Roots were observed for PRL during the first 9 d after germination. Haploid PRL was significantly shorter than diploid PRL from Day 1 (\( p = 0.0124 \)) onward, with haploids measuring 0.89 cm (±0.05) and diploids 1.05 cm (±0.05) the first day. Only after Day 2 was this relationship highly significant (\( p < 0.0001 \)), with haploid PRL measuring 1.91 cm (±0.06) and diploid PRL 2.77 cm (±0.06) on Day 2 (Table 1). The PRLs of haploids were normally distributed throughout all days. Distributions of diploid PRL values were normally distributed for Days 1 to 5 but skewed to the left after Day 6 (Fig. 2). Absolute differences in diploid and haploid PRL increased until Day 6: from 0.16 cm on Day 1 to 4.8 cm on Day 6. Differences ([(diploid PRL − haploid PRL)/haploid PRL]100) increased from
18.0% on Day 1 to 78.4%, the maximum difference, on Day 6 (Table 2). Although genotype was significant for the early time points (Days 1–5), it became nonsignificant after Day 6 ($p = 0.29$). The interaction between genotype and ploidy was not significant, except on Days 2 ($p = 0.051$) and 3 ($p = 0.045$). The experiment factor was significant across all days ($p < 0.0001$). The genotype $\times$ experiment interaction was not significant for any days, whereas the ploidy $\times$ experiment interaction was significant for later days, such as Days 7 ($p = 0.05$), 8 ($p = 0.009$), and 9 ($p = 0.002$). Correlations between different days of PRL measurement were all significant and ranged from $R = 0.21$ to 0.99, except for correlations of Day 1 with other Days 7 to 9 (Tables 3 and 4).

The PRL GR was measured by taking PRL measurements on all 9 d of growth after germination and fitting a linear regression for these data points. When analyzed for GR, ploidy was significant ($p < 0.0001$) (Table 1). Distributions for haploids were normal but left skewed for diploids (Fig. 3). Genotype ($p = 0.17$) and the genotype $\times$ ploidy interaction ($p = 0.07$) were not significant, while the experiment factor was significant ($p < 0.0001$). The only interactions that were significant for GR were the ploidy $\times$ experiment interaction ($p = 0.0002$). The GR of diploids was 1.68 cm/d ($\pm 0.03$), whereas haploids grew 0.91 cm/d ($\pm 0.03$) (Table 2).

For the analysis of the germination date, only the experiment factor was significant ($p < 0.0001$) (Table 1), which explains its significant impact on other traits. The average germination date for diploids was 1.26 d ($\pm 0.02$) and for haploids 1.23 d ($\pm 0.02$). Similarly, the average germination date for Wf9 was 1.24 d ($\pm 0.02$), and for PHZ51 it was 1.26 d ($\pm 0.02$). Average germination dates for different experiments, however, showed much more variation. Germination dates ranged from 1.00 d ($\pm 0.02$) to 1.74 d ($\pm 0.02$) for the different experiments.

Diploids exhibited more variation in root development. Some diploids exhibited a constant increase in PRL, whereas others ceased growth of the primary root and instead allocated energy to developing secondary roots (Fig. 4). If secondary roots were formed, they began to grow by Days 5 to 6.

### Exploring Criteria for Haploid–Diploid Discrimination

Receiver operating characteristic curves were analyzed and areas under the curve (AUC) were calculated to determine the performance of all selection methods. Youden’s $J$ statistic was computed as an additional indication of performance. Optimal criteria for selection were computed with the raw data, as well as for two scenarios, where haploid prevalence ($P$) was 10% or 20%. False negative rates (FNRs) and false positive rates (FPRs) were estimated for all cases. In both scenarios, heavier weight was placed on false negatives (see above). All selection method performances are summarized in Table 5.

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**Table 1. Analysis of primary root length by days after germination, growth rate (GR), and germination date (Germ).** Reported values are $p$ values, and significance was considered at $p = 0.05$.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
<th>GR</th>
<th>Germ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment (E)</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td></td>
</tr>
<tr>
<td>Genotype (G)</td>
<td>0.0011</td>
<td>0.0019</td>
<td>0.0004</td>
<td>0.0027</td>
<td>0.0177</td>
<td>0.2576</td>
<td>0.3939</td>
<td>0.972</td>
<td>0.4061</td>
<td>0.2786</td>
<td>0.3930</td>
</tr>
<tr>
<td>$E \times G$</td>
<td>0.0641</td>
<td>0.9174</td>
<td>0.8344</td>
<td>0.6881</td>
<td>0.8542</td>
<td>0.6608</td>
<td>0.7615</td>
<td>0.2585</td>
<td>0.1754</td>
<td>0.8398</td>
<td>0.5913</td>
</tr>
<tr>
<td>Ploidy (P)</td>
<td>0.0004</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
<td>$&lt;0.0001^{**}$</td>
</tr>
<tr>
<td>$E \times P$</td>
<td>0.0677</td>
<td>0.697</td>
<td>0.3887</td>
<td>0.8964</td>
<td>0.3199</td>
<td>0.0576</td>
<td>0.0501</td>
<td>0.0085</td>
<td>0.0022</td>
<td>0.0002</td>
<td>0.3939</td>
</tr>
<tr>
<td>$G \times P$</td>
<td>0.7287</td>
<td>0.0512</td>
<td>0.0454</td>
<td>0.259</td>
<td>0.3487</td>
<td>0.3576</td>
<td>0.2959</td>
<td>0.2508</td>
<td>0.1934</td>
<td>0.2197</td>
<td>0.0830</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
Method A: Trait Evaluation Not Adjusted for Germination

When PRL was not adjusted for germination date, selection was based on PRL measurements on a single day, and the day with the highest AUC was Day 9. The AUC values ranged from 0.58±0.054 (Day 2) to 0.81±0.019 (Day 9) for Days 2 to 9. If selection was performed on Day 9, the optimal criterion for selection would be PRL £ 11.35 cm and would result in a FNR of 5% and a FPR of 46% (Fig. 5a). If a scenario is considered with $P = 10\%$, the haploid selection criterion would be PRL £ 5.26 cm with a FNR of 6% and a FPR of 7%. If $P = 20\%$, then the optimal criterion for haploid selection would again be £11.35 cm but with a FNR of 1% and a FPR of 37%. Based on Youden’s J statistic, however, the highest value was obtained at Day 4. Youden’s J statistic values ranged from 0.19 (Day 2) to 0.53 (Day 3) for Days 2 to 9. The optimal criterion for haploid selection based on PRL measurements on Day 4 was £ 3.99 cm, resulting in a FNR of 12% and a FPR of 36%. If $P = 10\%$, the haploid selection criterion would be PRL £ 3.59 cm with a FNR of 3% and a FPR of 23%. When $P = 20\%$, the haploid selection criterion would be PRL £ 3.99 cm, producing a FNR of 2% and a FPR of 29%.

Method B: Trait Evaluation Adjusted for Germination

If PRL is adjusted for germination, selection was based on PRL measurements on a single day, and the day with the highest AUC was Day 9. The AUC values ranged from 0.58±0.054 (Day 2) to 0.81±0.019 (Day 9) for Days 2 to 9. If selection was performed on Day 9, the optimal criterion for selection would be PRL £ 11.35 cm and would result in a FNR of 5% and a FPR of 46% (Fig. 5a). If a scenario is considered with $P = 10\%$, the haploid selection criterion would be PRL £ 5.26 cm with a FNR of 6% and a FPR of 7%. If $P = 20\%$, then the optimal criterion for haploid selection would again be £11.35 cm but with a FNR of 1% and a FPR of 37%. Based on Youden’s J statistic, however, the highest value was obtained at Day 4. Youden’s J statistic values ranged from 0.19 (Day 2) to 0.53 (Day 3) for Days 2 to 9. The optimal criterion for haploid selection based on PRL measurements on Day 4 was £ 3.99 cm, resulting in a FNR of 12% and a FPR of 36%. If $P = 10\%$, the haploid selection criterion would be PRL £ 3.59 cm with a FNR of 3% and a FPR of 23%. When $P = 20\%$, the haploid selection criterion would be PRL £ 3.99 cm, producing a FNR of 2% and a FPR of 29%.

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Table 2. Average primary root length and growth rate with standard errors for haploids and diploids across and within genotypes. Days have been adjusted for germination date.

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary root length (cm)</th>
<th>Growth rate (cm/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>Diploid</td>
<td>2.79±0.06</td>
<td>7.09±0.08</td>
</tr>
<tr>
<td>Haploid</td>
<td>1.88±0.07</td>
<td>4.24±0.09</td>
</tr>
<tr>
<td>WF9 Diploid</td>
<td>3.01±0.09</td>
<td>7.34±0.12</td>
</tr>
<tr>
<td>WF9 Haploid</td>
<td>1.95±0.09</td>
<td>4.35±0.12</td>
</tr>
<tr>
<td>PHZS1 Diploid</td>
<td>2.57±0.08</td>
<td>6.84±0.11</td>
</tr>
<tr>
<td>PHZS1 Haploid</td>
<td>1.82±0.10</td>
<td>4.12±0.13</td>
</tr>
</tbody>
</table>

Table 3. Correlations of haploid primary root length between different days (adjusted for germination). Values above the diagonal are Pearson correlation coefficients, while those below the diagonal are $p$ values for significance.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.72</td>
<td>0.57</td>
<td>0.38</td>
<td>0.32</td>
<td>0.25</td>
<td>0.11</td>
<td>−0.08</td>
<td>−0.11</td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>&lt;0.0001</td>
<td>0.88</td>
<td>0.72</td>
<td>0.63</td>
<td>0.54</td>
<td>0.44</td>
<td>0.32</td>
<td>0.25</td>
<td></td>
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<tr>
<td>Day 3</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.91</td>
<td>0.85</td>
<td>0.78</td>
<td>0.71</td>
<td>0.63</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.96</td>
<td>0.91</td>
<td>0.86</td>
<td>0.81</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.97</td>
<td>0.94</td>
<td>0.90</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td>0.0038</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.98</td>
<td>0.96</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>0.2065</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.99</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>0.4525</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Day 9</td>
<td>0.3209</td>
<td>0.0048</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tr>
</tbody>
</table>

Table 4. Correlations of diploid PRL between different days (adjusted for germination). Values above diagonal are Pearson correlation coefficients, and below diagonal are $p$ values for significance.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 9</th>
</tr>
</thead>
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<tr>
<td>Day 1</td>
<td>0.81</td>
<td>0.59</td>
<td>0.44</td>
<td>0.32</td>
<td>0.21</td>
<td>0.15</td>
<td>0.13</td>
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<tr>
<td>Day 2</td>
<td>&lt;0.0001</td>
<td>0.88</td>
<td>0.73</td>
<td>0.60</td>
<td>0.49</td>
<td>0.43</td>
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<td>0.36</td>
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</tr>
<tr>
<td>Day 3</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.89</td>
<td>0.79</td>
<td>0.72</td>
<td>0.67</td>
<td>0.61</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.93</td>
<td>0.87</td>
<td>0.81</td>
<td>0.76</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.96</td>
<td>0.90</td>
<td>0.87</td>
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<td>Day 6</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.96</td>
<td>0.94</td>
<td>0.92</td>
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</tr>
<tr>
<td>Day 7</td>
<td>0.0702</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.96</td>
<td>0.94</td>
<td>0.92</td>
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<tr>
<td>Day 8</td>
<td>0.1697</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.98</td>
<td>0.96</td>
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<tr>
<td>Day 9</td>
<td>0.1292</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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highest AUC was found for Day 4. The AUC values ranged from 0.53 (±0.033) (Day 1) to 0.89 (±0.014) (Day 4) among Days 1 to 9. Youden’s J statistic values ranged from 0.15 (Day 1) to 0.62 (Day 4) among Days 1 to 9. If Day 4 was chosen for selection based on PRL measurements, the optimal criterion for haploid selection was PRL ≤ 5.47 cm, providing a FNR of 21% and a FPR of 17%. For P = 10%, the optimal criterion for haploid selection was PRL ≤ 5.11 cm, providing a FNR\textsubscript{10} of 3% and a FPR\textsubscript{10} of 11%. If P = 20%, the optimal criterion for haploid selection was PRL ≤ 5.80 cm, resulting in a FNR\textsubscript{20} of 3% and a FPR\textsubscript{20} of 18% (Fig. 5b).

Method C: Trait Evaluation for Growth Rate

A selection method using GR across all 9 d and adjusted for germination date resulted in an AUC of 0.81 (±0.020). The Youden’s J statistic was 0.59. If GR was used for selection, the optimal criterion for haploid selection was ≤1.52 cm/d with a FNR of 11% and a FPR rate of 30%. If P = 10%, the optimal criterion was ≤1.52 cm/d, and the FNR\textsubscript{10} dropped to 1% and the FPR\textsubscript{10} to 27%. If P = 20%, the optimal criterion was ≤1.55 cm/d, resulting in a FNR\textsubscript{20} of 2% and a FPR\textsubscript{20} of 25% (Fig. 5c).

Method D: Trait Evaluation by Support Vector Machine Learning

The SVM method utilized germination-adjusted PRL of a particular day, all prior days’ PRL measurements, and the growth rates. Best results with the SVM method were obtained on Day 3 after germination. The AUC was 0.90 (±0.019) for Day 3, which was higher than for any other selection method. Moreover, Youden’s J statistic (0.72) was higher than with any other method. By this method, any output (Y) value that is negative is predicted to be haploid, with smaller (more negative) values increasing the probability of haploidy. The optimal criterion in our study was a negative value ≤ −0.08. This criterion would result in a FNR of 14% and a FPR of 14%. If P = 10%, the optimal criterion would be ≤ −0.21, resulting in a FNR\textsubscript{10} of 2% and a FPR\textsubscript{10} of 9%. If P = 20%, the optimal criterion would be ≤ −0.08 with a FNR\textsubscript{20} of 3% and FPR\textsubscript{20} of 11% (Fig. 5d).

Discussion

Haploids and the effects of ploidy in general have been thoroughly studied (Chase, 1964; Guo et al., 1996; Miller et al., 2012). In maize, Randolph et al. (1944) observed a doubling in volume between diploid and tetraploid structures, such as cells, tissues, and organs. Tetraploid structures, although larger, contained a similar number of parts and appeared to have no deformities (Randolph et al., 1944). Chase (1964) hypothesized that maize haploids would
also exhibit a decrease in volume in similar proportions without deformities. However, he found that haploids were smaller than expected (approximately 11% under theoretical expectations). Although the reasons for this are unclear, it has been termed the odd-ploidy effect phenomenon (Guo et al., 1996). Reduced vigor of haploids compared with diploids observed in our study for PRL is consistent with these past findings.

Overall, using PRL to discriminate haploids from diploids has been shown to be a viable and practical option for selection when incorporating EC into DH programs. The selection methods developed here show similar, if not superior, performance compared with traditional R1-nj or oil-content-based haploid selection. The study of Chaikam et al. (2017) found similar results in radicle length of haploid seedlings and showed that pairing R1-nj selection with observations of certain traits such as radicle length, coleoptile length, number of lateral seminal roots, and presence of root hairs reduced the FPR to 9.4%, which was a 3.5-fold improvement when compared to R1-nj selection alone. Past experiments reported an average FPR of 25.2 to 40.7% and FNR of 12.7 to 51.7% using R1-nj selection (Chaikam et al., 2017; Melchinger et al., 2014). Moreover, there are some genotypes, such as those that contain the allele C1-l, that will inhibit the accumulation of anthocyanin, which is responsible for the marker characteristics of R1-nj (Paz-Ares et al., 1990). There are, in addition, genotypes that naturally create dark purple colored kernels, which mask marker characteristics, making selection by R1-nj equally impossible.

Selection based on oil content is a more recent method, depending on the availability of a high-oil haploid inducer. Selection based on oil content currently achieves an average FPR of 30.8% and a FNR generally below 15% (Melchinger et al., 2014). These rates may improve with the availability of inducers with higher oil content. Moreover, the use of any genetic-based marker, including R1-nj or oil content, requires the introgression of these markers into the inducer genome prior to the implementation of these selection methods in DH programs. This would not be required if reduced haploid vigor for PRL or other traits could be used for haploid–diploid discrimination.

Performance evaluation of each selection method (based on AUC and Youden’s J statistic, respectively) resulted in the following ranking from best to worst: Day 3 SVM analysis (0.90, 0.72), Day 4 PRL after adjustment for germination (0.89, 0.62), Days 1 to 9 GR after adjustment for germination (0.81, 0.59), Day 9 without adjustment for germination (0.81, 0.49), and Day 4 PRL without adjustment for germination (0.77, 0.53). When haploids represent 10% of the population, the SVM selection method captured 98% of all haploids, while the rate of incorrectly classified hybrids was much lower (9%) than for the other methods (up to

Fig. 5. False negative rate (FNR) and false positive rate (FPR) in relation to criterion values of several methods of selection, from worst to best performance: (a) primary root length (PRL) on Day 9 after setup, (b) PRL on Day 4 after germination, (c) growth rate (GR) of PRL during Days 1 to 9 after germination, and (d) support vector machine (SVM) analysis on Day 3 after germination.
Moreover, when haploids represent 20% of the population, the SVM method still represents the best selection method. Compared with standard methods of haploid selection, the SVM selection method improves on both FPR (16% for R1-nj and 22% for oil content) and FNR (11% for R1-nj and 13% for oil content) (Chaikam et al., 2017; Melchinger et al., 2014).

The only drawback to the SVM selection method is the necessity to measure PRL for the first 3 d. Because these days need to be adjusted for the germination date of each individual sample, it is a method that would be more difficult to incorporate into a high-throughput protocol. For a more streamlined protocol that retains early differentiation, implementing selection on Day 4 based on PRL that is not adjusted for germination may be the best option. This method requires only a single PRL measurement and can be accomplished on the same day for all samples, regardless of germination. The accuracy of selection is compromised, yet surprisingly it is still slightly better than standard selection methods. Compared with standard methods of haploid selection, the SVM selection method improves on both FPR (2% for R1-nj and 8% for oil content) and FNR (10% for R1-nj and 12% for oil content) (Chaikam et al., 2017; Melchinger et al., 2014).

It should be noted that the measurement of total root length instead of, or in addition to, PRL may result in a more efficient selection of haploids. Most of the variability in root development was observed in diploids, because some diploids allocated energy to growing secondary roots instead of the primary root in later days. The software ARIA (Pace et al., 2014), developed by the co-authors, which can measure up to 27 different root traits including PRL and total root length, may be useful to resolve this issue. If the selection process is based on total root length, the assumption would be that diploids are in general more vigorous and grow longer roots regardless of whether they are the primary or secondary roots. The software ARIA was not used because water droplets and glares reduced image quality. This issue may be overcome by constructing fully sealed plates from thicker plastic sheeting to allow above or vertical pouring of the medium. This would eliminate any unwanted empty space between the medium and front sheeting where interfering water droplets from high-humidity conditions tend to accumulate. Future studies may research these alternative plates, in addition to including automation of image capture, to further the likelihood of making this method high throughput.

Additionally, future studies need to confirm that there is minimal genotype × ploidy interaction once a broad range of genotypes is used. Although this interaction was insignificant in this study, these findings were expected because ploidy was measured on only two genotypes. When applying this method to breeding programs, it would be advisable to conduct a small training set to observe the potential variation in genotypes. Conducting a training set would be wise, regardless, to confirm that threshold values for haploid selection are optimal for the genotypes of interest.

The most impactful benefits for adding EC to a DH program is the potential to decrease generation time by approximately 6 wk and to increase the genome doubling rates as high as 90% (Barton et al., 2014). High doubling rates would reduce the resources needed for other steps of the DH procedure, such as the number of induction crosses and number of haploids to be planted in the field. Other benefits include more convenient and safer use of colchicine. Not only is it safer to handle colchicine in a gel form (compared with a liquid in traditional methods), but the total amount of colchicine needed would be reduced. The short exposure time required (up to 24 h) allows embryos to be placed in a single petri dish of shallow colchicine-containing media before being transferred to the large custom-built containers for root observation. In addition, there is also the benefit of reducing generation time by harvesting immature embryos rather than waiting for seed maturation. It is well documented that higher levels of colchicine exposure can stunt seedlings (Havas, 1937; Dermen and Brown, 1940). However, the effect may be equally observed among haploids and diploids, maintaining the relative differences in PRL.

References


