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RESEARCH

# Assessment of doubled haploid production efficiency in maize using sequential phenotypic marker screening

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**Abstract** Doubled haploid (DH) technology has become an important tool for accelerating maize breeding by enabling the rapid development of completely homozygous lines. However, breeding decisions depend not only on haploid induction rate or chromosome doubling success, but also on the cumulative efficiency of the entire DH pipeline. Despite its practical importance, information on material losses from induced seed to recovered DH line remains limited under routine breeding conditions. The aim of this study was to assess the efficiency of doubled haploid production in maize by quantifying material losses across successive stages of haploid induction, phenotypic selection, chromosome doubling, and final recovery of fertile DH lines. Fifty genetically diverse maize donor populations were pollinated with a maternal haploid inducer carrying the dominant phenotypic markers *R1-nj* and *Pl1*. Putative haploid seeds were initially identified using the *R1-nj* anthocyanin marker, followed by secondary validation using the *Pl1* root pigmentation marker. A total of 38,916 seeds were evaluated, of which 5,870 putative haploids were identified at the first screening stage, corresponding to a putative haploid selection rate of 15.08%. Secondary screening reduced this number to 3,316 putative haploids (8.52%). Following chromosome doubling, field evaluation, fertility screening, and the removal of non-uniform and electrophoretically segregating ears, 213 fertile putative $DH_0$ ears were retained, resulting in a final operational recovery efficiency of 0.55%. Seed storage protein electrophoresis of well-filled ears confirmed the genetic uniformity of selected DH lines, while low-seed $DH_0$ ears were retained as putative lines requiring further progeny evaluation for full confirmation. The results demonstrate that sequential phenotypic marker screening improves haploid identification accuracy and provide practical estimates of operational losses across the DH pipeline under applied breeding conditions.

**Keywords** *Zea mays* L. · Doubled haploid (DH) technology · *R1-nj* marker · *Pl1* marker · Chromosome doubling · Breeding efficiency

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## Introduction

The standard method for developing inbred maize lines is the pedigree method, which involves self-pollination of the breeding population for 6–8

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generations, accompanied by continuous selection of progenies that meet the breeder's objectives (Hallauer et al. 1988). Therefore, reducing the time and resources allocated to this lengthy process remains a major challenge for breeders. Haploid technology represents an efficient approach for the rapid development of completely homozygous lines in a significantly shorter time (Chaikam and Prasanna 2020). This approach involves the use of specific maize genotypes known as haploid inducers, which possess the genetic ability to induce maternal haploids, also referred to as matroclinous haploids (Khammona et al. 2025). Naturally occurring maternal haploids are extremely rare in maize, with classical studies reporting frequencies of approximately one haploid per 1,000 seeds (Randolph 1940; Chase 1949). The development of specialized maternal haploid inducers (Nanda and Chase 1966) transformed haploid production from a rare biological phenomenon into a practical breeding technology. Pollen from maternal haploid inducer lines is used to pollinate selected donor genotypes, resulting in the production of maternal haploids from the donor parent (Trampe et al. 2022).

Haploid induction rate is strongly influenced by the genetic constitution of the inducer. Several loci associated with haploid induction ability have been identified, including *qhir1* and *qhir8*, whose underlying genes contribute to the induction of maternal haploids and the variation in induction efficiency observed among inducer populations (Prigge et al. 2012; Kelliher et al. 2017; Liu et al. 2017; Zhong et al. 2019; Li et al. 2021).

The resulting haploids are generally male sterile and therefore require chromosome doubling to restore fertility. The most accessible and widely used method is colchicine treatment, which involves the application of aqueous colchicine solutions, an alkaloid extracted from autumn crocus (*Colchicum autumnale L.*) (Chalyk 2003; Chaikam et al. 2020). This process consists of several stages aimed at fixing desirable genotypes that are difficult and time-consuming to obtain through conventional selfing (Prasanna et al. 2012). Although genetic mechanisms of spontaneous haploid genome doubling have also been reported, their efficiency remains insufficiently optimized for large-scale application in breeding programs (Boerman et al. 2020; Foster et al. 2024).

A doubled haploid (DH) plant is formed when haploid cells (n), containing a single genome, undergo either spontaneous or artificially induced genome doubling. DH line development enables the rapid fixation of recombinant genotypes in fewer generations than traditional breeding methods (Szarejko and Forster 2006; Chang and Coe 2009; Geiger and Gordillo 2009). Doubled haploid (DH) technology has been successfully applied in more than 250 plant species (Forster and Thomas 2005) and has become a standard component of modern maize breeding programs. It is routinely integrated into both public and commercial breeding pipelines because it accelerates breeding cycles, improves selection efficiency, and reduces the time and resources required for line development compared with conventional pedigree breeding (Röber et al. 2005; Geiger and Gordillo 2009; Prasanna et al. 2012; Chaikam et al. 2019; Chaikam and Prasanna 2020; Indu et al. 2023).

The efficiency of individual stages within the maize DH pipeline varies considerably among studies and genetic backgrounds. The identification of haploid seeds and plants represents a critical step in doubled haploid production. The most widely used marker system is the *R1-navajo* (*R1-nj*) anthocyanin marker, which enables visual discrimination between haploid and diploid kernels at the seed stage. The *R1* gene, located on chromosome 10, encodes a basic helix–loop–helix (bHLH) transcription factor that acts together with the MYB transcription factor encoded by *C1* to regulate anthocyanin biosynthesis in maize kernels (Chandler et al. 1989; Ludwig et al. 1990; Cone et al. 1993a). In induction crosses, diploid hybrid kernels typically exhibit anthocyanin pigmentation in both the aleurone and embryo, whereas haploid kernels display pigmentation only in the aleurone layer because the embryo lacks the inducer genome (Nanda and Chase 1966; Geiger and Gordillo 2009). Previous studies demonstrated that *R1-nj*-based classification may produce substantial numbers of false positives, with false discovery rates exceeding 20% and reaching over 40% in some germplasm backgrounds (Röber et al. 2005; Chaikam et al. 2015, 2016).

To improve the accuracy of haploid identification, some maternal haploid inducers also carry the *Pl1* marker (Rotarenco et al. 2010). The *Pl1* gene, located on chromosome 6, encodes an R2R3-MYB transcription factor involved in the regulation of anthocyanin accumulation in roots and vegetative tissues (Taylor and Briggs 1990; Cone et al. 1993b; Pilu et al. 2003).

When present in inducer lines, *Pl1* serves as a complementary second-stage marker for distinguishing haploid and diploid seedlings based on anthocyanin pigmentation. Whereas *R1-nj* is used for initial kernel screening, *Pl1* enables validation of putative haploids at the seedling stage, thereby reducing misclassification errors during DH production (Chaikam et al. 2016).

Modern maternal haploid inducers typically exhibit haploid induction rates ranging from approximately 8% to more than 20% (Rotarenco et al. 2010; Prasanna et al. 2012; Almeida et al. 2020; Chen et al. 2024). However, visual haploid identification based on the *R1-nj* marker may be affected by incomplete marker expression and pigmentation inhibitors, resulting in false-positive classifications and overestimation of haploid frequency (Dermail et al. 2024; Khammona et al. 2025). Likewise, chromosome doubling and fertility restoration remain major bottlenecks, with substantial losses commonly occurring between haploid identification and recovery of fertile DH plants (Chaikam and Prasanna 2020). Because most studies report these stages separately, the cumulative efficiency of the entire DH production pipeline, from induced seed to recovered DH line, is less frequently quantified under practical breeding conditions. Collectively, these technical limitations directly influence the practical implementation of DH technology because they affect the amount of biological material, labor, field space, and financial resources required to recover a target number of doubled haploid lines.

Despite its widespread adoption in modern maize breeding, breeders in Eastern Europe, including the Republic of Moldova, still rely largely on conventional inbreeding methods and remain cautious regarding the practical efficiency of DH technology (Borozan et al. 2023; Musteata 2024). In addition to the more complex logistics involved, one constraint frequently discussed by breeders is that recombination events become fixed earlier in DH-derived lines than in conventionally selfed populations. Extracting DH lines from $F_2$ populations rather than directly from $F_1$ plants has been recommended to increase the number of recombination events before fixation (Bernardo 2009). In some cases, $F_2$ induction resulted in ~ 50% more recombination events, but no significant difference in the means for yield or plant height between the two generations, suggesting that the additional recombination did not immediately translate into superior elite lines (Sleper and Bernardo 2016). In tropical maize, the use of $F_1$ generation was recommended due to a superior balance between time and genetic variability (de Oliveira Couto et al. 2019).

Although the principles of DH technology are well established, most studies report individual components of the DH pipeline, such as haploid induction rate, marker-based haploid identification, chromosome doubling efficiency, or fertility restoration (Prasanna et al. 2012; de Oliveira Couto et al. 2019; Boerman et al. 2020; Maqbool et al. 2020; Trentin et al. 2020; Baleroni et al. 2021; Aboobucker et al. 2022; Dermail et al. 2024). Comparatively less information is available on cumulative material losses from the initial population of induced seeds to the final recovery of fertile $DH_0$ ears under routine breeding conditions. Such information is essential for breeding programs seeking to estimate the amount of donor material, field space, labor, and laboratory resources required to generate a target number of DH lines (Longin et al. 2007; Grüning et al. 2025).

Therefore, the aim of this study was to assess the efficiency of doubled haploid production in maize by quantifying material losses across successive stages of the DH pipeline, from haploid induction and phenotypic marker-based selection to chromosome doubling and final recovery of fertile DH lines. This evaluation may provide practical insights for local breeding programs by offering a clearer understanding of the entire DH process and enabling breeders to make informed adjustments in resource allocation, workflow organization, and breeding strategies.

## Materials and methods

The research was conducted during 2022–2023 at the experimental field of the Faculty of Agricultural, Forestry and Environmental Sciences, Technical University of Moldova, located in Chișinău, Mircești Street 50. The soil of the experimental field is classified as typical chernozem, with a high degree of anthropogenic influence.

The study was conducted under temperate climatic conditions. During the 2022 and 2023 growing seasons, precipitation was below the long-term average and temperatures were higher than normal; therefore, supplementary drip irrigation was applied to

avoid severe moisture stress and ensure normal plant development.

As biological materials, 50 temperate maize donor populations were used for haploid induction. The donor material consisted of $F_2$ populations derived from commercial maize hybrids adapted to Eastern European environmental conditions. The use of $F_2$ populations was consistent with recommendations that doubled haploid extraction from $F_2$ rather than $F_1$ generations increases the opportunity for recombination before fixation of homozygosity (Bernardo 2009). Each donor population was planted in a single-row plot 5 m long with 0.7 m row spacing, corresponding to an experimental unit area of approximately 3.5 $m^2$. The 50 donor populations were arranged in five blocks of ten donor rows each.

To obtain haploid seeds, donor populations were pollinated with pollen from a haploid inducer carrying the dominant color markers *R1-Navajo* (*R1-nj*) and *Pl1*. The inducer population was established in four adjacent rows located alongside the donor plots to provide a continuous source of pollen during flowering. The haploid inducer used in this study was a MAGIC (*Multiparent Advanced Generation InterCross*) population developed at Iowa State University and transferred under a material transfer agreement in 2022. The population originated from an eight-way intercross among elite maternal haploid inducer lines developed from crosses between the RWS/RWK76 inducer background and exPVP/public maize inbred lines. The parental inducer lines were fixed for the major haploid induction genes *mtl* and *zmdmp*, carried the dominant phenotypic markers *R1-nj* and *Pl1*, and exhibited haploid induction rates of approximately 12–16%. The breeding scheme and genetic composition of this germplasm have been described previously (Chen et al. 2024, 2025).

Controlled pollinations were performed using bulk pollen collected from multiple flowering plants within the haploid inducer population. Donor plants served as female parents, while the haploid inducer was used as the pollen donor. Controlled pollinations were conducted using standard maize hand-pollination techniques, including isolation of tassels and ears with paper bags and manual transfer of pollen (Hallauer et al. 1988).

Each donor population was pollinated on 3–5 ears depending on flowering synchronization and seed set. After harvest, putative haploid kernels identified from all donor populations were combined and processed collectively during the subsequent screening and chromosome doubling stages; therefore, the experiment was designed to estimate overall operational DH production efficiency across a broad donor set rather than to compare genotype-specific haploid induction rates. Haploid selection was performed sequentially using dominant phenotypic markers. The first stage of selection was based on the *R1-nj* marker, which enables rapid and efficient identification of putative haploid seeds at the seed stage. Hybrid seeds exhibit anthocyanin pigmentation in both the embryo and endosperm, whereas haploid seeds display pigmentation only in the endosperm while the embryo remains uncolored (Chaikam et al. 2015).

The second stage of selection was carried out at the seedling stage using the *Pl1* root marker, which induces anthocyanin pigmentation in seedling roots and serves as an effective trait for identifying false putative haploid plants after the initial *R1-nj*-based selection. Seedlings expressing anthocyanin pigmentation in the roots were classified as diploids and discarded, while non-pigmented seedlings were retained as putative haploids for subsequent chromosome doubling procedures (Khammona et al. 2025).

Chromosome doubling of putative haploid plants was performed using a 0.125% colchicine solution supplemented with 0.5% dimethyl sulfoxide (DMSO), applied via microinjection at the 3–4 leaf stage. Approximately 0.1 mL of solution was injected per plant directly into the region of the shoot apical meristem (Chalyk 2003). Following chromosome doubling treatment, seedlings were transplanted immediately to the field nursery and maintained under standard agronomic practices with regular irrigation. No acclimatization period was applied because colchicine-treated haploid seedlings frequently develop necrosis, loss of apical meristem activity, and reduced vigor within a few days after treatment, making delayed transplantation increasingly difficult. Immediate transplantation enabled seedlings to establish under field conditions while they were still physiologically viable.

Following transplantation to the field nursery, putative haploid plants were subjected to an additional phenotypic verification step based on anthocyanin pigmentation of vegetative tissues. Plants were periodically evaluated during vegetative growth for the presence of anthocyanin coloration on stems and leaf sheaths. Because expression of inducer-derived

anthocyanin markers may occur in vegetative tissues, plants exhibiting anthocyanin pigmentation were classified as false putative haploids and discarded.

At flowering, plants producing fertile tassels were self-pollinated under controlled conditions using paper isolation bags to prevent contamination from foreign pollen. Following pollination, ears remained covered until harvest.

At harvest, ears exhibiting seed color segregation were discarded as probable hybrids or the result of pollen contamination. To further assess genetic uniformity, selected well-filled putative $DH_0$ ears containing more than 100 seeds were subjected to polyacrylamide gel electrophoresis (PAGE) of seed storage proteins (zeins) in an acidic buffer system (Batiru et al. 2023; Sidorova et al. 2023). This threshold ensured sufficient seed material for both electrophoretic evaluation and subsequent maintenance of the corresponding line. For each ear, six seeds were analyzed individually. Zein proteins were extracted from the endosperm fraction and separated by vertical PAGE under acidic conditions in the presence of urea using a VS20 WAVE Maxi vertical electrophoresis system (Cleaver Scientific, UK). Aliquots of 20 µL protein extract were loaded into individual wells, and electrophoresis was performed at a constant voltage of 500 V for 5 h. Gels were stained with Coomassie Brilliant Blue R-250 and scored for zein banding patterns. Uniform electrophoretic spectra were considered indicative of genetic homogeneity and consistent with doubled haploid origin, whereas ears showing segregating spectra were classified as heterogeneous and discarded.

Ears with low seed set were retained as putative $DH_0$ lines because reduced fertility is common following chromosome doubling in maize haploids and does not, by itself, indicate non-DH status. These ears originated from fertile self-pollinated plants, exhibited no visible seed segregation, and were advanced for subsequent progeny testing to confirm genetic uniformity.

Experimental data were collected at each stage of the haploid induction and selection process. Due to the large number of haploid seedlings processed during chromosome doubling (> 3,000 plants), individual survival rates following colchicine treatment, field transplantation, and pre-flowering mortality were not recorded separately. These stages were managed operationally as bulk populations.

Each surviving plant was treated as an individual breeding unit and evaluated for marker expression, fertility restoration, and seed production.

The final efficiency assessment was therefore based on the number of fertile putative $DH_0$ ears recovered after the complete screening and selection process.

The efficiency of doubled haploid production was determined as the percentage ratio between the number of retained fertile putative $DH_0$ ears and the total number of seeds evaluated after haploid induction (hybrid seeds + selected haploid seeds):

$$E_{DH(\%)} = \frac{N_{DH}}{N_{Total}} \times 100 \quad (1)$$

where: $E_{DH}$—efficiency of doubled haploid production (%); $N_{DH}$—number of retained fertile putative $DH_0$ ears; $N_{Total}$—total number of seeds evaluated after haploid induction (hybrid seeds + putative haploid seeds).

The use of the total number of screened seeds as the denominator was intended to quantify the operational efficiency of the entire DH production process rather than the efficiency of individual stages such as haploid induction, chromosome doubling, or fertility restoration. This metric reflects the amount of initial biological material required to recover a fertile putative $DH_0$ line and therefore provides practical information for breeding program planning and resource allocation.

Because donor populations were processed collectively after the initial selection stages, genotype-specific effects could not be quantified. Therefore, the reported values represent overall operational DH production efficiency rather than genotype-specific performance.

## Results and discussion

Initial screening using the *R1-nj* marker identified putative haploid kernels based on the absence of anthocyanin pigmentation in the embryo. Although hybrid seeds were excluded from subsequent DH production steps, they were included in the calculation of operational recovery efficiency because all seeds generated by induction crosses constituted the initial biological material that required harvesting, handling, and screening.

Following pollinations with the haploid inducer population, different levels of *R1-nj* expression were observed among donor genotypes, indicating genotype-specific variation in marker penetrance (Fig. 1).

Donor populations exhibited marked differences in seed pigmentation intensity, which complicated the visual identification of haploid kernels. Because material from all donor populations was pooled after the initial screening stage, the frequency of marker inhibition and the proportion of affected genotypes could not be quantified. Nevertheless, the observed variation highlights the limitations of relying exclusively on *R1-nj* for haploid identification.

The anthocyanin marker *R1-nj*, widely used for visual haploid selection, may be affected by partial or complete inhibition of pigmentation expression, as well as by naturally occurring anthocyanin pigmentation in the pericarp of certain genotypes, resulting in misclassification and false-positive haploid identification (Chaikam et al. 2015; Trentin et al. 2022; Khammona et al. 2025). As shown in Fig. 1, variation in seed pigmentation intensity among donor populations highlights the influence of donor genetic background on the reliability of *R1-nj*-based haploid identification. Pigmentation inhibitors are especially common in flint and sweet maize germplasm and may completely suppress *R1-nj* expression, making haploid identification difficult or even impossible in some donor backgrounds (Paz-Ares et al. 1990; Chaikam et al. 2015; Trentin et al. 2022). This limitation has been widely documented in previous studies. Misclassification rates based on *R1-nj* expression have ranged from 0 to 45.2%, depending on the donor germplasm (De La Fuente et al. 2018). Prigge et al. (2011) reported that *R1-nj* was most effective in single-cross germplasm, but considerably less reliable in landraces, where more than half of the evaluated populations exhibited misclassification rates exceeding 30%. Similarly, Röber et al. (2005) found that the proportion of verified haploids reached 89.6% in dent germplasm, but only 48.0% in flint germplasm, indicating substantially lower classification accuracy in flint backgrounds. In tropical maize germplasm, false discovery rates exceeding 24% have been reported, with some donor populations showing values greater than 40% (Chaikam et al. 2016). These findings highlight the strong influence of donor genetic background on the reliability of *R1-nj*-based haploid identification and support the use of additional validation markers and confirmation steps.


**Fig. 1** Variation in *R1-nj* anthocyanin marker expression among pollinated donor populations: 1—strong expression with clear differentiation between putative haploid and hybrid kernels; 2—intermediate expression; 3—partial inhibition of anthocyanin pigmentation complicating haploid identification; 4—complete suppression of pigmentation preventing visual discrimination between putative haploid and hybrid kernels

In this context, the use of complementary markers, such as pigmentation of embryonic roots and leaf sheaths at the seedling stage, allows additional verification of the selected material and improves the accuracy of haploid identification. Previous studies have shown that the red root pigmentation marker (*Pl1*) is rarely found in maize germplasm and can therefore be effectively used as a complementary marker to *R1-nj*, particularly in populations where *R1-nj* marker expression is inhibited. Consequently, combining multiple sequential phenotypic markers represents an effective strategy to reduce selection errors and improve the final efficiency of doubled haploid line production (Chaikam et al. 2016).

Based on the considerations mentioned above, at the second selection stage, the putative haploid seeds were planted in trays and evaluated for anthocyanin pigmentation in the roots, determined by the presence of the *Pl1* gene (Fig. 2).

Seedlings exhibiting pink pigmentation in the roots were discarded as false putative haploids, while non-pigmented seedlings were subsequently treated with a colchicine solution and transplanted to the field.

In addition to root pigmentation, plants were monitored during vegetative growth for anthocyanin


**Fig. 2** Identification of maternal haploids based on the *Pl1* color marker system. Left: hybrid seedling expressing anthocyanin pigmentation (*Pl1* marker present); right: putative haploid seedling lacking anthocyanin pigmentation (*Pl1* marker absent)

coloration of stems and leaf sheaths. Because expression of inducer-derived anthocyanin markers may occur in vegetative tissues, plants exhibiting anthocyanin pigmentation were classified as false putative haploids and discarded. This additional verification step helped eliminate plants that may have escaped detection during the initial root-based screening (Khammona et al. 2025).

At flowering, plants producing fertile tassels (i.e., capable of producing pollen) were identified. These plants were subjected to controlled self-pollination and covered with paper isolation bags to prevent contamination from foreign pollen. After pollination, the ears remained covered until harvest.

At harvest, ears showing seed color segregation were discarded, as these were considered hybrids or the result of pollen contamination. Ears of doubled haploid lines exhibited no seed pigmentation and varied considerably in seed set, ranging from a single seed per ear to complete seed filling (Fig. 3). Each fertile putative $DH_0$ plant was self-pollinated individually and typically produced a single ear, which was harvested and maintained separately as an individual genotype.

Data analysis showed that a total of 5,870 putative haploid seeds were selected from harvested ears. The haploid frequency estimated using the *R1-nj* marker (15.08%) was within the range commonly reported for modern maternal haploid inducers, which generally varies from 8% to more than 20% depending on inducer genotype, donor germplasm, and environmental conditions (Prasanna et al. 2012; Chaikam et al. 2019; Almeida et al. 2020; Trentin et al. 2022; Chen et al. 2024).

Following germination, 3,316 haploid embryos did not express root pigmentation and were classified as putative haploids based on the absence of the *Pl1* marker (Table 1).

The reduction from 15.08% putative haploids identified by *R1-nj* to 8.52% after *Pl1* screening highlights the importance of secondary marker systems for eliminating false putative haploids. Approximately 43.5% of the *R1-nj*-selected kernels were discarded during *Pl1* validation, indicating a substantial frequency of false positives. Similar observations were reported by Chaikam et al. (2016), who found an average false discovery rate of 24.2% for *R1-nj*-based classification, with some donor populations exceeding 40%, and demonstrated that the red root marker (*Pl1*) substantially improves the recovery of true haploids. In addition, *Pl1*-based classification showed a stronger agreement with true ploidy status and lower misclassification rates than *R1-nj*, confirming its value as an effective secondary screening tool for improving haploid identification accuracy.

At harvest, 218 putative $DH_0$ ears were recovered. Of these, 15 well-filled ears containing more than 100 seeds were subjected to seed storage protein electrophoresis (Fig. 4). Uniform zein banding patterns were observed in 10 ears, whereas 5 ears (33.3%) showed

**Fig. 3** Putative $DH_0$ ears recovered after self-pollination

**Table 1** Operational efficiency of doubled haploid production in maize

| DH production stage | Number | % of total seeds | % retained from previous stage | % loss from previous stage |
|---|---|---|---|---|
| Total seeds subjected to haploid screening | 38,916 | 100.00 | – | – |
| Putative haploid seeds (*R1-nj* screening) | 5,870 | 15.08 | 15.08 | 84.92 |
| Putative haploid seedlings (*Pl1* screening) | 3,316 | 8.52 | 56.49 | 43.51 |
| Fertile putative $DH_0$ ears retained | 213 | 0.55 | 6.42 | 93.58 |
| Final operational recovery efficiency | 0.55% | – | – | – |

segregating spectra and were discarded. Seed storage protein electrophoresis (PAGE) of maize zeins has long been used for genetic identification, seed purity assessment, and characterization of inbred germplasm (Kahrıman et al. 2022; Sidorova et al. 2023). Therefore, the occurrence of segregating zein spectra among putative $DH_0$ ears provides evidence of residual genetic heterogeneity and supports the use of electrophoretic validation as a complementary tool for confirming line uniformity. The detection of segregating protein spectra in 5 of the 15 analyzed ears indicates that sequential phenotypic screening based on *R1-nj* and *Pl1* markers, although effective, does not completely eliminate non-uniform material. Similar observations have been reported following molecular, cytological, and biochemical verification of putative doubled haploids, highlighting the importance of complementary validation procedures for eliminating false positives and ensuring genetic uniformity of recovered lines (Chaikam and Prasanna 2020; Dermail et al. 2024). Consequently, phenotypic marker systems should be regarded as efficient screening tools rather than definitive confirmation of doubled haploid status.

A limitation of the present study is that doubled haploid status was inferred using sequential phenotypic screening based on the *R1-nj* and *Pl1* markers, complemented by zein PAGE analysis, rather than direct molecular or cytological verification. Although this approach represents a practical and cost-effective strategy for large-scale DH production and is routinely used in regional breeding programs, a certain

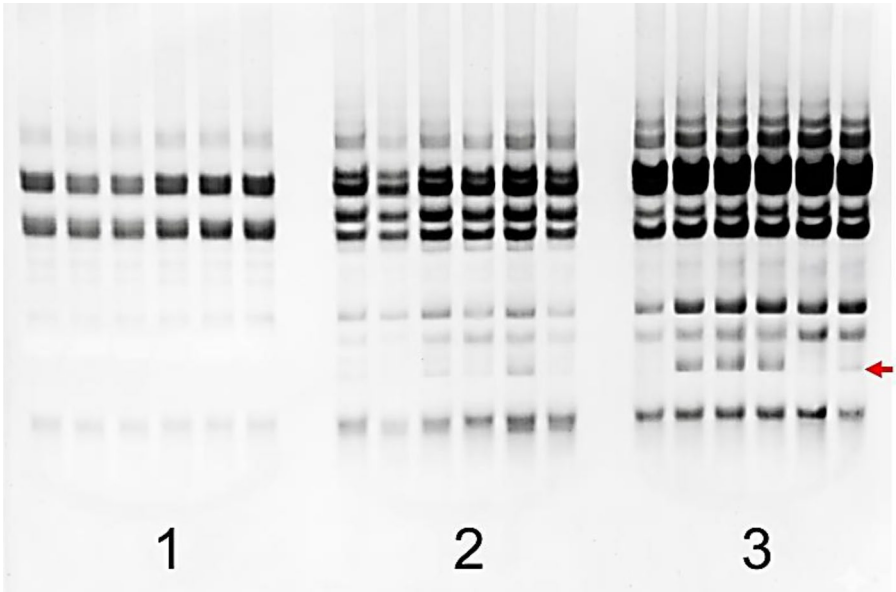


**Fig. 4** Use of seed storage protein (zein) polymorphism in $DH_0$ seeds to confirm homozygosity: 1—homozygous; 2–3—segregating spectra. Arrow indicates polymorphic band associated with segregation

proportion of false-positive or incompletely fixed genotypes cannot be entirely excluded. Future studies should incorporate SSR or SNP genotyping, flow cytometry, or chromosome counting to independently validate DH status, quantify residual heterozygosity, and further refine estimates of overall DH production efficiency.

After this elimination, 213 fertile putative $DH_0$ ears remained. The largest proportional loss occurred between the putative haploid seedling stage and the recovery of fertile $DH_0$ ears, where only 6.42% of the selected haploids ultimately produced selfed seed. This finding confirms that chromosome doubling, plant survival, and fertility restoration remain the principal bottlenecks in maize DH production. The reduction from 3,316 putative haploids to 213 retained $DH_0$ ears primarily resulted from mortality following colchicine treatment, transplant losses, incomplete chromosome doubling, sterility, and elimination of non-uniform or segregating ears. Because ears with very low seed numbers could not be reliably validated by electrophoretic analysis, they were retained as putative $DH_0$ lines. Consequently, the recovered material should be regarded as putative $DH_0$ lines pending further confirmation in the $DH_1$ generation through progeny evaluation, including assessment of phenotypic uniformity, absence of segregation, fertility, and stability of seed traits. Additional biochemical or molecular marker analyses may be applied when sufficient seed is available. Although PAGE analysis of zeins provided a practical and economical approach for detecting segregation among well-filled $DH_0$ ears, its resolution is lower than that of modern DNA-based marker systems and residual heterozygosity cannot be completely excluded. Therefore, complementary molecular validation may further improve the accuracy of DH status confirmation, particularly in cases involving spontaneous chromosome doubling, incomplete doubling, or chimeric plants that may produce fertile ears despite not representing true doubled haploids (Battistelli et al. 2013; Chaikam et al. 2019). Future studies should quantify survival rates at intermediate stages of the DH pipeline to identify opportunities for improving chromosome doubling efficiency and reducing operational losses (Fig. 4).

Thus, the proportion of putative haploid seeds identified through *R1-nj* screening was 15.08% of the total seed population, whereas 8.52% of the total seeds remained classified as putative haploids after the additional *Pl1* screening stage. The overall operational success rate, calculated as the number of retained fertile putative $DH_0$ ears relative to the initial number of evaluated seeds, was 0.55%. Similar cumulative losses have been reported in other DH production studies. de Oliveira Couto et al. (2019) recovered only 27 fertile DH lines from 415,979 induced seeds after successive stages of haploid identification,

chromosome doubling, and self-pollination. Likewise, Cengiz et al. (2020) obtained 27 DH lines from 15,911 induced seeds despite initially identifying 3,012 putative haploids. Although direct comparisons are complicated by differences in germplasm, induction systems, and chromosome doubling protocols, these studies consistently demonstrate that only a small proportion of the initial induction material ultimately results in fertile homozygous lines. Together with the present results, they emphasize the importance of evaluating cumulative recovery efficiency across the entire DH pipeline rather than relying solely on haploid induction rate or chromosome doubling success as isolated indicators of performance.

Unlike conventional estimates of haploid induction or chromosome doubling efficiency, the present metric describes the cumulative operational efficiency of the entire DH production process. By considering all seeds subjected to screening in the denominator, it provides a practical estimate of the biological material and resources required to recover fertile $DH_0$ lines under breeding conditions.

Under the conditions of this study, an induced donor ear produced approximately 200 kernels on average. Based on the 38,916 seeds evaluated, this corresponds to approximately 195 induced ears. Because 213 fertile putative $DH_0$ ears were ultimately recovered, approximately one induced donor ear was required to obtain one fertile putative $DH_0$ line. This operational relationship provides a practical planning tool for breeding programs because it directly links the amount of induction material required to the expected number of DH lines recovered, information that is rarely reported, but is essential for estimating field, greenhouse, and laboratory requirements.

The efficiency observed in the present study may be further improved through the use of modern inducers with higher haploid induction rates. Several contemporary inducer populations exceed 20% induction efficiency (Almeida et al. 2020; Chen et al. 2024, 2025), and continued inducer development has successfully combined higher induction rates with improved agronomic performance and marker expression (Trentin et al. 2020; Ulyanov et al. 2022). However, excessively high induction rates may also compromise inducer vigor, pollen production, and seed set, requiring a balance between induction efficiency and agronomic performance (De La Fuente 2015). Additional gains may be achieved through improved chromosome doubling protocols. Our unpublished observations suggest that increasing colchicine concentration to 0.2% enhanced the frequency of male-fertile plants, which is consistent with previous reports demonstrating improved chromosome doubling efficiency without substantial reductions in plant survival (Chaikam et al. 2020). Furthermore, ongoing efforts to exploit spontaneous chromosome doubling may provide additional opportunities to improve the overall efficiency of maize DH production in the future (Foster et al. 2024).

## Conclusions

A total of 38,916 seeds derived from crosses between 50 maize donor populations and a maternal haploid inducer were evaluated throughout the doubled haploid production pipeline. Initial screening based on the *R1-nj* marker identified 5,870 putative haploid seeds (15.08%), while secondary validation using the *Pl1* marker reduced this number to 3,316 putative haploids (8.52%), confirming the value of sequential phenotypic marker screening for eliminating false positives and improving haploid identification accuracy. Following chromosome doubling, field evaluation, fertility screening, and the elimination of non-uniform material, 213 fertile putative $DH_0$ ears were recovered, corresponding to an overall operational recovery efficiency of 0.55%.

The largest losses occurred between haploid selection and $DH_0$ recovery, indicating that chromosome doubling, plant survival, and fertility restoration remain the principal bottlenecks in maize DH production. Nevertheless, the results demonstrate that candidate doubled haploid lines can be generated within only two generations and provide a practical estimate of the biological material required for DH line development under breeding conditions. The operational efficiency metrics reported in this study may serve as useful benchmarks for planning resources and optimizing doubled haploid breeding programs

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carried out by B.G. and C.D. The first draft of the manuscript was written by B.G. and C.D. Manuscript review and editing were performed by C.G. Research supervision was provided by B.G. and C.G. Funding acquisition was secured by B.G. All authors read and approved the final manuscript.

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**Data availability** The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

**Declarations**

**Conflict of interest** The authors declare no competing interests.

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