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# Generation of maize (*Zea mays*) doubled haploids via traditional methods

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**ABSTRACT**

Commercial maize hybrid production has corroborated the usefulness of producing inbred lines, however, the delivery of new lines has always been a major time constraint in breeding programs. Traditional methods for developing inbred lines typically require 6-10 generations of self-pollination to obtain sufficient homozygosity. To bypass the time and costs associated with the development of inbred lines, doubled haploid (DH) systems have been widely adopted in the commercial production of maize. Within just two generations, DH systems can create completely homozygous and homogeneous lines. A typical maize DH system, utilizing anthocyanin markers *R1-nj* or *Pl1* for haploid selection, is described in this protocol.

**Keywords: Maize - Doubled Haploids - Haploid selection**

## INTRODUCTION

After the discovery and exploitation of heterosis, yield increases and advancements in large scale commercial maize production encouraged the use and spurred the demand of this commodity in the U.S. and worldwide (Shull, 1908; Shull, 1909). Breeders are continuously pressed to rapidly deliver lines with new traits, coupled with enhanced yield, especially as the global human population increases. Given that traditional methods for developing inbred lines typically require 6-10 generations of self-pollination to obtain sufficient homozygosity (Hallauer et al., 2010), doubled haploid (DH) technology provides an attractive alternative to delivering inbred lines in only a few generations. Currently, DH technology is well known to be an efficient and reliable means of advancing maize breeding programs. This technology is highly utilized in commercial breeding programs worldwide (Schmidt, 2003; Seitz, 2005; Chen et al., 2009).

Doubled haploids are created when a haploid (n) plant undergoes genome doubling due to the exposure to a doubling agent or mitotic inhibitor, the most common being colchicine, resulting in genetically uniform diploid offspring with two identical genomes (2n). The most common method of producing haploid plants in maize DH line production is *in-vivo* maternal haploid

induction, which requires a specific inducer genotype. When an inducer is crossed to a maize donor plant, progeny will segregate into diploid (2n) and haploid (n) classes. Haploids still contain a normal triploid endosperm, however, allowing for proper germination. Current maternal haploid inducers are all derived from Stock 6, which was found to display a maternal haploid induction rate of 1-2% (Coe, 1959). With the advent of Stock 6 and after decades of improvement, inducers with higher haploid induction rates made the *in-vivo* maternal doubled haploid system feasible for large scale maize production (Geiger, 2009). Research programs created improved *in-vivo* maternal haploid inducers based on Stock 6, including RWS, KEMS, WS14, and MHI, among many others with higher haploid induction rates (Röber et al., 2005; Shatskaya et al., 1994; Lashermes and Beckert, 1988; Eder and Chalyk, 2002). These inducers (used as the male parent) are crossed to a donor genotype or population (used as the female parent), currently resulting on average in 8% haploid progeny (Prigge et al., 2011). A dominant anthocyanin gene, *R1-navajo* or *R1-nj* (Chase and Nanda, 1965; Nanda and Chase, 1966; Greenblatt and Block, 1967; Neuffer et al., 1997), was incorporated into inducer genotypes, which allows for visual selection of mature haploid kernels. Once haploids are selected, they are germinated before exposing them to a doubling agent. Typically, they are treated with colchicine before being transferred to the field to reach maturity. Haploids displaying fertility restoration, usually in a chimeric pattern, can be self-pollinated to create a doubled haploid line.

This publication provides a detailed protocol describing how to create maize doubled haploids using the most common approaches.

## GENERATION OF MAIZE DOUBLED HAPLOIDS

Doubled haploid technology is a popular approach to develop inbred lines in just two generations. The recent review by Liu et al. (2016) provides an extensive list of uses for DH technology, which include: rapid development of inbred lines, marker-assisted backcrossing, gene stacking, population improvement, exploitation of genetic resources, among many others. DH technology does not only save time and resources, but has many other benefits. For instance, this is the only method that will arrive at a completely homozygous and homogeneous line, and fulfills all requirements necessary for Plant Variety Protection (PVP) (Liu et al., 2016). In addition, selection during the haploid stage is more efficient and precise, especially when used in addition with molecular markers (Liu et al., 2016). Since haploids only contain one set of chromosomes, it is easier to observe and select certain alleles of interest. Similarly, natural selection on haploids can be utilized to remove harmful mutations (Chang and Coe, 2009).

This protocol involves four major steps: 1) production of haploids by *in-vivo* maternal haploid induction, 2) selection of haploid kernels or seedlings, 3) genome doubling by application of a doubling agent to haploid seedlings, and 4) self-fertilization of successfully doubled haploids.

### *Materials*

- Donor population (female parent)
- Maternal *in-vivo* haploid inducer genotype (male parent) containing *R1-nj*
  *Note*: A full list of inducers can be found in Liu et al. (2016)
- Germination and potting equipment (pots, plug trays, soil, etc.)
- Colchicine and dimethyl sulfoxide
- Syringes for injection of colchicine solution
- Access to a greenhouse
- Pollination equipment (shoot and tassel bags, garden snips, etc.)

***Production of haploids by in-vivo maternal haploid induction***

1) Select a donor population to induce and plant this in the field, usually between mid-May and early June or when weather is appropriate. Along with the donor population, 1-2 delays of the inducer genotype should be planted as well. Plant approximately one inducer plant for every 6 donor plants.

   *Donor populations are usually an $F_1$ or $F_2$ from any combination of lines. Maize inducers are generally considered genotype independent. Although there are some exceptions, they maintain the ability to induce haploids when combined with mostly all donor populations.*

2) When flowering begins (generally 9-10 weeks after emergence), take precautions to maintain controlled pollinations. De-tassel donor plants to avoid contamination and accidental self-pollinations. The inducer genotype (male parent) is used to pollinate a donor genotype (female parent).

   *Keeping in mind that on average only 8% of progeny will be haploid, it is important to make a sufficient number of pollinations. See the "Anticipated Results" section for more information on calculating how many induction crosses are necessary.*

***Selection of haploid kernels or seedlings***

3) Allow 55-60 days for full maturation or stage R6, approximately 30% moisture (Nielsen, 2001) and dormancy of kernels before harvesting.

4) After kernels are dried, identify and select haploid kernels based on the dominant anthocyanin marker, *R1-nj*, during the shelling process (Figure 1). In diploid kernels, *R1-nj* is expressed as a red/purple coloration in the aleurone (outermost layer of endosperm, 3n) and the scutellum (embryo, 2n). Since haploid kernels have a triploid (3n) endosperm, the aleurone expresses this coloration, while the haploid embryo (1n), possessing only the donor genotype, does not express this coloration. Thus, a haploid

kernel is identified based on coloration on the crown, and simultaneously the absence of coloration in the embryo.

*Other alternative markers/methods for haploid kernel selection include oil content (using high-oil inducers), near-infrared spectroscopy, or seed weight. See 'Background information' for details.*

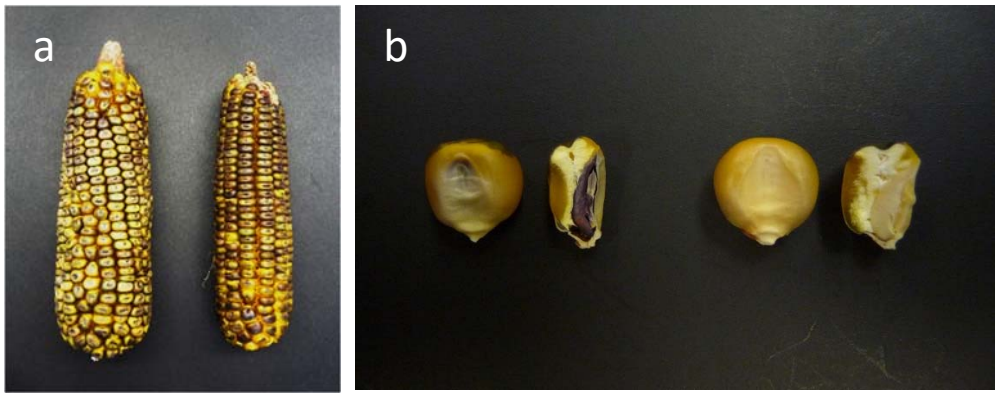


Figure 1: a) Ears resulting from pollinations by inducers carrying the *R1-nj* anthocyanin marker. b) Whole and cross-sectioned views of diploid (left) and haploid (right) mature kernels. The anthocyanin marker, *R1-nj*, produces the red/purple coloration observed on the crown of both diploid and haploid as well as the coloration in the diploid embryo.

5) If the donor population contains a dominant anthocyanin inhibitor gene in the genetic background, such as *C1-I*, *C2-Idf*, or *In1-D*, it will block the ability to accurately identify haploid kernels by the *R1-nj* marker (Coe, 1994). Another anthocyanin marker observed as red/purple coloration of roots, *Pl1* (*Purple1*), can be used in this case (Sastry, 1970). If possible, sort out clear diploids based on *R1-nj* and germinate the remaining potential haploids to view root coloration for more accurate selection. Germinate seeds in multi-plug trays (200 or 400 plugs per tray), as these trays use limited soil and root balls are easily viewed within one week. Due to having limited soil, however, trays can dry out quickly. It might be necessary to water trays several times per day to keep plugs moist. After approximately one week, haploids can be selected based on root coloration. Gently pull out the seedling from the plug trays, trying to limit damage or tearing of roots (squeezing the individual plug from beneath can help loosen the root ball). If desired, wash the soil off of the roots to better see the coloration, but this is usually not necessary. Seedlings with red/purple roots are discarded as diploids, or false positives of the *R1-nj* kernel color selection (Figure 2a), while seedlings with white roots are selected as haploids (Figure 2b). Depending on your transplanting system, repot the seedlings to the respective pots and continue cultivation until the 2-3 leaf stage is reached.

*It is important to prevent light exposure to roots by covering seedlings with enough soil and keeping roots under the tray, because there is a potential for light-induced anthocyanin production. If the donor population carries a recessive*

*light-dependent pl1 allele in the genetic background, tissues exposed to light develop a "sun-red" phenotype (Emerson, 1921; Briggs, 1966; Coe et al., 1988).*


Figure 2: a) Diploids displaying red/purple coloration in roots due to the presence of the dominant *Pl1* gene inherited from the inducer genotype. b) Haploid seedlings carry only the maternal genome and do not carry the *Pl1* gene, resulting in colorless, or white roots.

***Genome doubling by application of a doubling agent to haploid seedlings***

6) Assuming haploid kernels were selected based on *R1-nj*, germinate selected haploid seeds in plug trays in a greenhouse 2-3 weeks prior to transplanting to the field. If haploids were selected based on *Pl1*, skip to the next step.

   *Since doubling rates (percentage of haploids that display male and female fertility) usually range between 20-25% and success rates (percentage of haploid seeds that result in a DH line) usually range from 8-10%, it is important to plant an adequate number of haploids to ensure the desired number of DH lines is reached. See the "Anticipated Results" section for more information.*

7) Once seedlings have 2-3 collared leaves (usually 10-12 days after planting) inject approximately 100 ul of 0.125% colchicine solution with 0.5% dimethyl sulfoxide (DMSO) into the stalks, or at 3-5 mm above the apex using a 1 ml (25G) sterile syringe (Figure 3) (Zabirova et al., 1996; Eder and Chalyk, 2002). Make sure the seedlings are well watered before treatment, as watering should be avoided for some hours after injection. Before starting the injection brush with your arm over the seedlings to remove excess water sitting in the whorl of the leaves. Start applying colchicine from the middle of tray and work outward to avoid reaching over already treated seedlings, turn the tray around and do the other half the same way. The leaves form a column

which is over-filled with the colchicine solution when injection is done successfully. After treatment, the seedlings are kept out of direct sunlight for some hours.

*The goal of the injection is to expose the basal meristem to colchicine because flowering tissues (tassel and silks) will be generated from this meristematic region. As mentioned, colchicine-treated haploids will be chimeric (not all tissues will be doubled), and it is vital that the flowering tissues are successfully doubled.*

*Colchicine has to be handled with caution – always wear PPE. The colchicine solution is sometimes dyed blue with the addition of vegetable dye (for example Color Coat ™ Blue from Becker Underwood) to aid the researcher in visualizing spills as a safety precaution. This avoids unnecessary prolonged contact with colchicine in the incident of an accidental spill. If skin comes into contact with the solution wash with plenty of water. The chemical is bought in small batches, to avoid unnecessary weighing of the powder (for example 1 g is sufficient for 800ml of solution, and about 7000-8000 injections). The solution has to be kept in a dark bottle as colchicine is susceptible to light. Colchicine solution can be stored at 4°C for 2-3 weeks. All plastic ware, tissues, syringes that come in contact with colchicine solution are autoclaved and disposed of by incineration. Left over solution is collected by Environmental Health and Safety Offices, or only authorized personnel for proper disposal.*

Figure 3: Injection of colchicine solution at the V3 stage. The goal is to make the injection a few mm above the meristematic region and within the center column. Usually injecting at an angle (as pictured) is a good method to target the center column.

***Self-fertilization of successfully doubled haploids***

8) After colchicine treatment, transplant the seedlings to the field (usually 1-2 days after injection). If conditions warrant, water seedlings after transplanting.

   *Transplanting is highly stressful especially for haploid plants, so being extremely responsive to the agronomic needs of the plantlets is a key to success.*

9) After a few weeks of growth, diploids that were mis-categorized or treated as a haploid will be obvious at this point. These plants will be significantly taller and have an overall greater vigor. The diploids usually show typical growth patterns, whereas haploids have narrow, more erect leaves, which sometimes displays white stripes (Chase, 1969). Diploids will also show red/purple coloration in the stalk, whereas haploids will not (Figure 4). Remove the diploid plants, because if left in the field, these plants can compete for water/resources and can also shade weaker haploid plants.


Figure 4: a) Haploids will show narrow erect leaves with colorless stalks. b) Diploids display vigorous growth and increased height, as well as red/purple coloration within the stalk.

10) Once shoots are exposed, cover them with a shoot bag until pollen begins to shed. If haploids display fertility restoration, it will usually be sectorial due to the chimeric pattern of genome doubling (Kato, 2002) (Figure 5a). The treatment of colchicine will only restore fertility when, by chance, the genome of cells which will produce flowering tissues is doubled. When pollen is shedding, tap anthers onto a paper bag for direct pollen collection. If there are only a few anthers that do not shed their pollen upon collection, it may be necessary to cut the anthers to release pollen (Figure 5b). If silks

are not emerged at the time of pollen shed, it may also be necessary to cut back the tip of the husk leaves in order to uncover receptive silks. Carefully disperse collected pollen over the silks. Isolate the pollinated ear by covering it with a pollination bag. Observe haploid plants on a daily basis since pollinations can be repeated up to two times if more fertile anthers emerge.

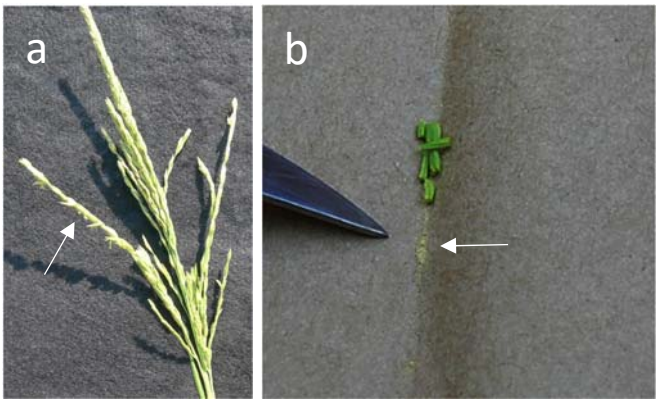


Figure 5: a) Haploid tassels will usually display sectorial sterility. The arrow indicates a tassel branch with restored fertility. b) It is important to collect any pollen possible, even if it involves cutting anthers to release pollen. The arrow points to the small amount of pollen released from a single cut anther.

**COMMENTARY**

**Background Information**

Since the introduction of DH technology to the maize industry, researchers have been consistently working for almost 60 years to improve the efficiency of this system (Chang and Coe, 2009). As a result, there are several alternative techniques that were developed. Generally, however, these alternatives did not become standardized due to the lack of efficiency, inferior end products, or the burden of extra labor.

The generation of haploids, for instance, has been successfully accomplished by several methods. Although alternatives exist, however, using an *in-vivo* maternal haploid inducer is typically the standard method in maize because it has been the most successful (Liu et al., 2016). One alternative is using an *in-vivo* paternal haploid inducer, which produces 1-2% haploid progeny due to the mutation of the gene *ig1* (*indeterminate gametophyte*) (Kermicle, 1969; 1971; 1994). If mutants are homozygous for the *ig1* mutation, gametophytes exhibit a prolonged phase of nuclear free divisions, which creates egg cells with abnormalities including those without a nucleus (Evans, 2007). Upon fertilization, haploids which contain the maternal cytoplasm and only one set of the paternal chromosomes are produced. Although this system is sometimes used for converting an inbred line to its cytoplasmic male sterile (CMS) form

(Pollacsek, 1992; Schneerman et al., 2000), it is not used in standard breeding programs due to low haploid induction rates.

Haploids can also be generated by anther-culture, but this procedure is an *in-vitro* process that requires much more resources and is genotype dependent (Maluszynski et al., 2003). Even after extensive research to optimize anther-culture procedures (Kuo et al., 1978) and to find genetic stocks that are responsive (Genovesi and Collins, 1982; Dieu and Beckert, 1986; Petolino and Jones, 1986), this procedure is not widely used in commercial production of maize. Unfortunately, most elite lines are non-responsive to this protocol (Maluszynski et al., 2003).

Not only are there alternative methods to creating haploids, there are alternative markers that can be used in place of *R1-nj* for selection of haploids. As mentioned, there are other methods of selection of mature kernels including oil content, near-infrared spectroscopy, and seed weight (Liu et al., 2016). The most reliable and accurate of these alternative selection methods is oil content, scoring above 90% accurate (Liu et al., 2016). High-oil inducers contain elevated levels of oil and because oil is primarily stored in the embryo, haploid kernels will contain less oil as compared to diploid kernels. For instance, non-destructive nuclear magnetic nesonance (NMR) measurements show that oil content on average is 5.26% for diploid kernels as compared to 3.42% for haploid kernels (Chen and Song, 2003). Other markers are observed in the seedling stage. As mentioned, there is another anthocyanin marker, *Pl1* (Sastry, 1970), that can also be utilized if selection based on *R1-nj* is error-prone. If the donor population contains a dominant anthocyanin inhibitor gene, such as *C1-I*, *C2-Idf*, or *In1-D*, it will block the ability to identify haploid kernels by the *R1-nj* marker (Coe, 1994), making the use of Pl1 necessary. Although *Pl1* could be used exclusively, it is usually only used in these cases where an alternative marker is necessary due to the additional labor involved with growing out all seedlings to observe root coloration.

Similarly, although colchicine is the most commonly used mitotic inhibitor, there are many other types that can be used and are less toxic, which include herbicides such as pronamid, APM, trifluralin, and oryzalin. These alternatives have likely not become standardized due to conflicting findings. For instance, these herbicides have been shown to be effective chromosome doublers (Häntzschel and Weber, 2010). Other studies however, show that doubling rates for these herbicides was not significantly different than spontaneous doubling rates (Vanous, 2011). It has also been found that the mitotic inhibitor can be successfully applied at different growth stages. For example, seedlings can also be soaked in a colchicine-containing solution shortly after germination, explained in Deimling et al. (1997). Soaking may result in a higher percentage of fertile haploids as compared to the traditional method of injection (Eder and Chalyk, 2002), but additional wash steps add to the labor involved, and the large volume of hazardous waste increases unnecessary handling of this carcinogen.

**Critical Parameters**

1. The success rate of doubling by colchicine injection may be influenced by temperature following injection. It is important to maintain the temperature at 25°C because higher temperatures can decrease doubling rates (Barow, 2006).
2. Maintaining optimum agronomic conditions for haploids is an important factor in haploid fertility. For instance, although male sterility is more common, stressed haploids may also exhibit female sterility.
3. Self-fertilization of successfully doubled haploids can sometimes be difficult due to the sectorial sterility of the tassel. It is important to pollinate whenever pollen is available. If the quality of a pollination is in question, a second pollination can be done the following day if more pollen is released.

**Troubleshooting**

| Problem | Possible Cause | Solution |
|---|---|---|
| Poor induction crosses | Poor weather conditions during pollination and grain fill. | Make many more induction crosses than necessary.<br><br>Provide many delays to avoid hot or dry phases of weather that may affect pollen quality. |
| Mis-characterized haploid | Poor sorting skills | Improve lighting of work space and practice often. |
| Male and/or female sterility in haploids | Poor agronomic conditions and/or extreme heat after colchicine injections | Do not allow haploids to endure stressful conditions for extended periods of time.<br><br>Control the temperature at 25°C after colchicine injections |

**Anticipated Results**

Doubled haploid procedures come with a high percentage of unsuccessful attempts that the researcher must anticipate if expected to arrive at a certain number of DH lines. For instance, induction crosses arrive at an average of only 8% haploid progeny (Prigge et al., 2011). In addition, not all haploids will arrive at a successful DH line. As mentioned, doubling rates (percentage of haploids that display male and female fertility) usually range between 20-25% and success rates (percentage of haploid seeds that result in a DH line) usually range from 8-10%. Assuming it is desired to arrive at 10 DH lines, one can work backwards and calculate the number of initial induction crosses needed (Figure 6).

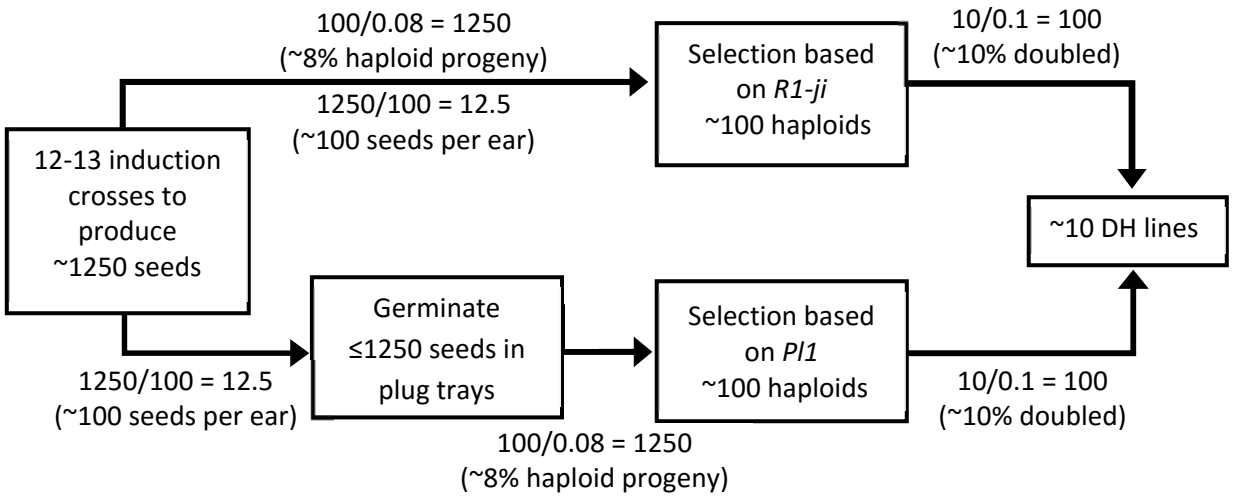


Figure 6: Diagram shows how to calculate the number of initial induction crosses necessary considering the desired end product is 10 DH lines. Calculations are shown for both traditional methods based on selection using *R1-nj* (top path) or traditional methods based on selection using *Pl1* (bottom path).

**Time Considerations**

The doubled haploid system in maize takes two generations to complete. The first generation is used to make induction crosses, or create haploid progeny. During the second generation, the haploid seedlings will be doubled and self-pollinated, thereby creating the doubled haploid line. These two generations will both likely take place during the growing season. If necessary, the first generation can be completed in the greenhouse or at a winter nursery location. It is not recommended that the second generation be grown in a greenhouse since optimum agronomic conditions are essential for restored fertility of colchicine-treated haploids.

For the second season, plan to plant the selected haploid kernels in the greenhouse by the end of April or early May if weather for the region allows field planting around mid-May. It takes approximately 10-20 days after emergence to reach the V3 stage at 350 GDUs (Neild, 1990). This will be the point at which colchicine can be injected and plantlets can be transplanted to the field. Pollination can be expected to come 6-7 weeks after transplanting.

**ACKNOWLEDGEMENT** (mandatory for NIH, optional for all others)

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