# Doubled Haploid

## Technology in Maize Breeding: Theory and Practice

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Headquartered in Mexico, the International Maize and Wheat Improvement Center (known by its Spanish acronym, CIMMYT) is a not-for-profit agriculture research and training organization. The center works to reduce poverty and hunger by sustainably increasing the productivity of maize and wheat in the developing world. CIMMYT maintains the world's largest maize and wheat seed bank and is best known for initiating the Green Revolution, which saved millions of lives across Asia and for which CIMMYT's Dr. Norman Borlaug was awarded the Nobel Peace Prize. CIMMYT is a member of the CGIAR Consortium and receives support from national governments, foundations, development banks, and other public and private agencies.



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**Abstract:** This manual is primarily intended for maize breeders in national agricultural research systems and small and medium enterprise seed companies in developing countries who would like to better understand and utilizes the doubled haploid (DH) technology in breeding programs. It is a compilation and consolidation of knowledge accumulated through scientific contributions of several maize geneticists and breeders worldwide as well as protocols successfully developed (in collaboration with the University of Hohenheim, Germany) and being used by the CIMMYT Global Maize Program in DH line development, especially in Mexico. An overview of the utility and applications of DH technology in maize breeding is presented first in the manual, followed by chapters on *in vivo* maternal haploid induction using haploid inducers, haploid kernel detection using anthocyanin markers, chromosome doubling of haploids, deriving DH seed from colchicine-treated plants, integrating molecular markers in DH-based breeding pipeline, DH in commercial maize breeding, and finally, access to tropicalized haploid inducers and DH service on cost-recovery basis to CIMMYT partners.

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# Contents

# 1. Doubled Haploid (DH) Technology in Maize Breeding: An Overview

***BM Prasanna***

**Introduction**

A "doubled haploid" (DH) is a genotype formed when haploid (*n*) cells successfully undergo either spontaneous or artificially induced chromosome doubling. Chase (1947, 1951, 1952, 1969) pioneered the studies on maize monoploids (synonymous to haploids, in the case of maize) and the use of DH lines in breeding. The DH technology shortens the breeding cycle significantly by rapid development of completely homozygous lines (in 2–3 generations), instead of the conventional inbred line development process, which takes at least 6–8 generations to derive lines with ~99% homozygosity (Forster and Thomas, 2005; Geiger and Gordillo, 2009; Chang and Coe, 2009).

Chase initially relied on spontaneous haploid induction and doubling, which was not quite conducive (due to very low frequency) to commercial application. The foundation for *in vivo* haploid induction using haploid inducers was laid when Coe (1959) described "a line of maize with high haploid frequency" of 2.3%, designated as "Stock 6." This genetic stock served as a founder for an array of inducers with higher haploid induction rates (HIR = number of kernels with haploid embryo divided by all kernels investigated) through the subsequent efforts of maize geneticists worldwide.

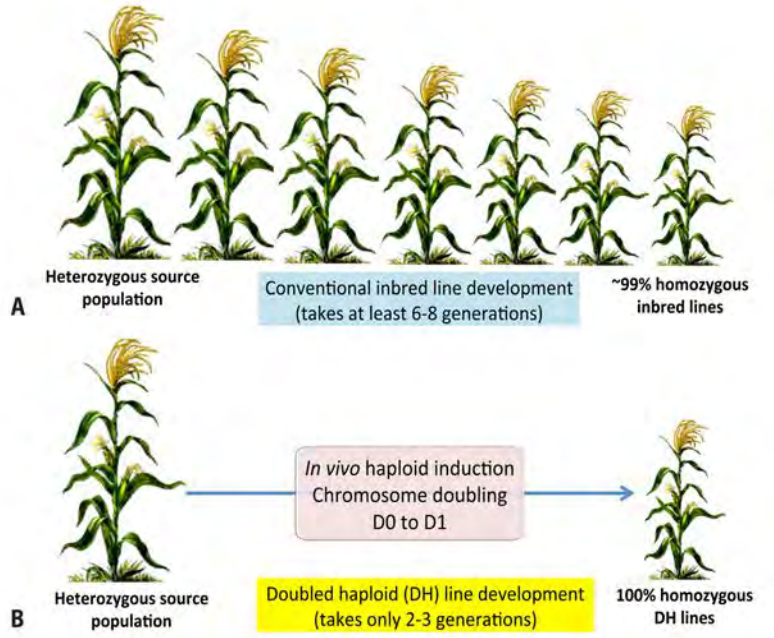


**Figure 1.** Number of generations to reach genetic purity (homozygosity) through: (A) conventional inbreeding; (B) doubled haploid technology.

Although DH lines in maize have been produced by several institutions using either *in vitro* or *in vivo* methods, the *in vitro* methods had very limited success due to non-responsiveness of many maize genotypes, besides the need to have a good laboratory and skilled staff. In contrast, *in vivo* haploid induction-based DH line development in maize is relatively easier, thanks to the efforts made by the maize geneticists in identifying "haploid inducer genetic stocks" (Coe, 1959; Coe and Sarkar, 1964), further incorporating an anthocyanin color marker in the inducer genetic backgrounds to facilitate easy identification of haploids at both the seed and seedling stages (Nanda and Chase, 1966; Greenblatt and Bock, 1967; Chase, 1969), and deriving new haploid inducers with higher HIR.

The DH technology in maize breeding, based on *in vivo* haploid induction, is recognized worldwide as an important means for enhancing breeding efficiency. In the last 10-15 years, the technology has been well adapted by several commercial maize breeding programs in Europe (Schmidt, 2003), North America (Seitz, 2005), and more recently in China (Chen et al., 2009), almost as soon as haploid inducer lines became available for temperate environments (Prigge and Melchinger, 2011). However, several of the maize breeding institutions in the public sector, as well as small and medium enterprise (SME) seed

companies in tropical maize growing countries in Latin America, sub-Saharan Africa and Asia, have lagged behind (Prasanna et al., 2010; Kebede et al., 2011). This may be due to several factors, including inadequate awareness about the DH technology, lack of access to the tropicalized haploid inducers, or lack of relevant "know-how" for effectively integrating DH in breeding programs. The purpose of this manual is, therefore, to introduce the theory and practice of DH technology in maize breeding.

**Why DH in maize breeding?**

The DH technology offers an array of advantages in maize genetics and breeding (Röber et al., 2005; Geiger, 2009; Geiger and Gordillo, 2009); salient among these are that it:

(1) Significantly shortens the breeding cycle by development of completely homozygous lines in two generations;
(2) Simplifies logistics (Geiger and Gordillo, 2009), including requiring less time, labor, and financial resources for developing new breeding lines; the time and resources thus saved could be potentially channelized for implementing more effective selections and for accelerated release of elite cultivars;
(3) Enables greater efficiency and precision of selection (Röber et al., 2005; Geiger and Gordillo, 2009), especially when used in combination with molecular markers and year-round nurseries;
(4) Accelerates product development by allowing rapid pyramiding of favorable alleles for polygenic traits influencing maize productivity and stress resilience, which are otherwise difficult and time-consuming to combine in adapted germplasm using conventional breeding practices;
(5) Perfectly fulfills the requirements of DUS (distinctness, uniformity, and stability) for plant variety protection due to the complete homozygosity and homogeneity of DH-based parental lines (Geiger and Gordillo, 2009);
(6) Reduces the effort for line maintenance (Röber et al., 2005);
(7) Can, in combination with molecular markers, facilitate access to the germplasm present within either the female or the male parental lines of hybrid cultivars (Heckenberger et al., 2005); and
(8) Provides opportunities for undertaking marker-trait association studies, marker-based gene introgression (Forster and Thomas, 2005), functional genomics, molecular cytogenetics, and genetic engineering (Forster et al., 2007; Wijnker et al., 2007).

***In vivo* maternal haploid induction-based DH development**

***Haploid induction***

The haploid inducers are specialized genetic stocks which, when crossed to a diploid (normal) maize plant, result in progeny kernels in an ear with segregation for diploid (2*n*) kernels and certain fraction of haploid (*n*) kernels due to anomalous fertilization. Kernels with a haploid embryo have a regular triploid (3*n*) endosperm, and therefore, these kernels are capable of displaying germination similar to those kernels with a diploid embryo (Coe and Sarkar, 1964).

The *in vivo* maternal haploid induction scheme at present relies on the presence of a dominant anthocyanin color marker, referred as *R1-Navajo* (*R1-nj*), that expresses in the aleurone (the outermost layer of the maize endosperm) as well as in the embryo (scutellum) in the haploid inducer, unlike the source populations, which do not usually have any anothocyanin coloration in the embryo or the endosperm. Thus, *R1-nj* as a dominant color marker helps in differentiation of monoploid/haploid (*n*) kernels (with no expression of purple/red colored anthocynanin in the scutellum, but with the typical crown-coloration on the endosperm), from the diploid (2*n*) kernels (with expression of anthocyanin in both the endosperm and scutellum) (Nanda and Chase, 1966; Greenblatt and Bock, 1967; Chase, 1969). Normal colorless kernels are the result of either selfing or contamination due to outcrossing. However, it must be noted that the expression of the *R1-nj* color marker can vary significantly depending on the

genetic background of the source genotype (in which maternal haploids have to be induced), the genetic background of the haploid inducer, as well as environmental factors (Chase, 1952; Röber et al., 2005; Kebede et al., 2011; Prigge et al., 2011).

***Temperate haploid inducers:*** A number of haploid inducer lines with high HIR and for commercial use have been derived over the years, with Stock 6 as the founder; these include: (1) **KMS** (Korichnevy Marker Saratovsky) and **ZMS,** both derived from Stock 6 (Tyrnov and Zavalishina 1984, cited in Chebotar and Chalyk, 1996); (2) **WS14**, developed from a cross between lines W23*ig* and Stock 6 (Lashermes and Beckert, 1988); (3) **KEMS** (Krasnador Embryo Marker Synthetic), derived from a cross (Shatskaya et al., 1994); (4); **MHI** (Moldovian Haploid Inducer), derived from a cross KMS × ZMS (Eder and Chalyk, 2002); (5) **RWS** (Russian inducer KEMS + WS14), descendant of the cross KEMS × WS14 (Röber et al., 2005); (6) **UH400,** developed at University of Hohenheim from KEMS (cited in Chang and Coe, 2009); (7) **PK6** (Barret et al., 2008); (8) **HZI1**, derived from Stock 6 (Zhang et al., 2008); (9) **CAUHOI**, derived at China Agricultural University from a cross between Stock 6 and Beijing High Oil Population (Li et al., 2009), and (10) **PHI** (Procera Haploid Inducer), derived from a cross between MHI and Stock 6 (Rotarenco et al., 2010).

The temperate inducers UH400, RWS, and RWS × UH400 were successfully employed for haploid induction and DH line development in CIMMYT's tropical and subtropical source germplasm from 2007 to 2011, although these temperate inducers are poorly adapted to tropical lowland conditions (Prigge et al., 2011). However, efficient and large-scale production of DH lines in tropical maize-growing environments using temperate haploid inducers could be severely constrained as these inducers display poor vigor, poor pollen production, poor seed set, and high susceptibility to tropical maize diseases.

***Tropicalized haploid inducers:*** Since 2007, CIMMYT Global Maize Program has been intensively engaged in optimization of the DH technology especially for the tropical/subtropical maize growing environments, in partnership with the University of Hohenheim, Germany. Tropically adapted inducer lines (**TAILs**; with 8–10% HIR) have been developed through this collaboration (Prigge et al., 2011). Experimental evaluation of the first-generation TAILs in two environments (Agua Fría and Tlatizapan in Mexico) over two seasons consistently resulted in average HIR ranging from 9% to 14%. A single-cross hybrid haploid inducer (with high HIR) has been developed using a sub-set of TAILs. The tropicalized haploid inducers are now available for sharing with interested institutions for research or commercial use under specific terms and conditions (http://www.cimmyt.org/en/about-us/media-resources/recent-news/1399-now-available-tropicalized-maize-haploid-inducer-lines). The availability of TAILs is expected to significantly enhance the efficiency of DH line production, increasing seed set and rates of induction, and reducing the costs of inducer line maintenance and seed production.

***Pathway for DH development and scope for further refinement***

It must be noted that efficient DH development is dependent not only on access to tropicalized haploid inducers with high HIR, but also on a number of other important steps in the DH production pipeline. The salient steps in DH development are: (1) crossing the source population (usually a hybrid generated using desired lines or F2 derived by selfing of the hybrid) as female parent with pollen of the haploid inducer; (2) identification of haploid kernels (at the dry seed stage) using the anthocyanin color marker; (3) germination of the haploid seeds; (4) safe application of colchicine or any other effective chromosome doubling agent to the haploid seedlings; (5) proper agronomic management of D0 seedlings and derivation of D1 (DH) seed by self-pollinating D0 plants; and (6) further selection and utilization of DH lines in breeding programs. The manual, in the subsequent chapters, provides both theoretical and practical details for each of the above steps. Some important steps that are further being refined through ongoing research in different institutions worldwide are highlighted below.

***Haploid identification:*** Although the *R1-nj*-based haploid identification scheme is, in general, quite effective, it is not without a pitfall. Presence of dominant anthocyanin inhibitor genes (such as *C1-I*, *C2-Idf,* and *In1*-D) in the source population or donor genome (Coe, 1994) or dosage effects can sometimes make this marker scheme ineffective. CIMMYT's elite germplasm is currently being surveyed to determine in what proportion the seed color marker will function, permitting efficient haploid seed detection. Currently, it appears that *R1-nj* color expression is inhibited in only about 8% of crosses of haploid inducers with diverse source populations.

The use of haploid inducers with anthocyanin genes *B1 (Booster1)* and *Pl1* (*Purple1*) that result in sunlight-independent purple pigmentation in the plant tissue (coleoptile and root) was found suitable for cases where haploid sorting is not possible at dry seed stage (Rotarenco et al., 2010). In this case, a pigmented coleoptile or root in the early developmental stage indicates diploid state, while the non-pigmented seedlings could be designated as haploids (Geiger and Gordillo, 2009; Rotarenco et al., 2010). Although CIMMYT has a few backcross populations that combine the root coloration marker with the *R1-nj* gene, the HIR, agronomic stability, and utility of this alternative marker scheme in DH production need to be established.

To avoid possible misclassification of haploids due to poor expression of anthocyanin color marker in the dry seed, Rotarenco et al. (2007) proposed haploid identification based on kernel oil content, determination of which can be potentially automated using nuclear magnetic resonance (NMR)-based techniques. Li et al. (2009) developed CAUHOI, a Stock 6-derived inducer with ~2% HIR and high kernel oil content (78 g $kg^{-1}$), that allows identification of haploids based on both lack of *R1-nj* conferred scutellum coloration and low embryo oil content. This novel approach looks promising, but its reliability and applicability for high-throughput DH production in tropical genetic backgrounds remains to be investigated. Jones et al. (2012) examined the utility of Near-infrared spectroscopy to differentiate haploids from hybrid maize kernels after maternal haploid induction.

***Chromosome doubling:*** Several institutions, including CIMMYT, currently use colchicine as a chromosome doubling agent (or mitotic inhibitor) in DH production, as spontaneous duplication of chromosomes occurs at a very low rate (Chase, 1969; Deimling et al., 1997). However, treatment with colchicine is not always completely effective, and sectoral diploidization of male and/or female inflorescences can occur. More importantly, colchicine is highly carcinogenic, requiring very careful handling and safe disposal after use. Herbicides such as pronamid, APM, trifluralin, and oryzalin have been reported to be efficient as mitotic inhibitors (Häntzschel and Weber, 2010). These are less expensive and less toxic than colchicine and are easier to handle and dispose of safely. Several commercial breeding companies apply proprietary artificial chromosome doubling treatments that are less toxic and safer than colchicine (Geiger and Gordillo, 2009).

***Agronomic management:*** Optimal agronomic management of the colchicine-treated D0 seedlings, first in the greenhouse and later in the field, is highly crucial for the success of DH line development, as discussed in detail in chapter 5 of the manual. Optimization of irrigation regime, fertilizer application, possible mechanization of operations, and effective management of weeds, diseases, and insects are crucial for minimizing stress on the D0 plants and improving the success rates of DH line production. In addition to proper agronomic management, the soil and climatic conditions at the DH operations site should be optimal.

**Mechanism(s) underlying maternal haploid induction**

As explained in detail in chapter 2 of this manual, several studies have been undertaken since the 1960s (reviewed by Eder and Chalyk, 2002; Geiger and Gordillo, 2009) to understand the biological mechanism(s) underlying *in vivo* maternal haploid induction. Although some important leads are available, the exact mechanism(s) behind maternal haploid induction are yet to be fully understood. This has not, however, limited large-scale derivation of DH lines and utilization of DH parental lines in developing and deploying commercial maize cultivars, especially by the major commercial maize breeding programs.

Genetic analyses of maternal haploid induction revealed polygenic control of the trait (Lashermes and Beckert, 1988; Deimling et al., 1997; Röber et al., 2005). Quantitative Trait Loci (QTL) mapping for *in vivo* haploid induction ability suggested that the trait is controlled by one or a few major QTL and several small-effect and/or modifier QTL. A major QTL on chromosome 1 (*qhir1,* in bin 1.04) explained up to 66% of the genetic variance for haploid induction ability in three populations involving a non-inducer parent and the HIR-enhancing QTL (Prigge et al. 2012). Identification and validation of breeder-ready markers for this major QTL and marker-assisted introgression of the favorable allele could potentially speed up the development of improved tropicalized haploid inducers with high HIR and local adaptation.

**DH technology and molecular markers, makes a very powerful combination**

Because DH technology offers a faster way to obtain completely homozygous lines, it can save significant time and resources for implementing genetic studies and/or molecular breeding projects, including:

1. Developing genetic maps (Chang and Coe, 2009; Forster et al., 2007), which is one of the widespread applications of DH populations in many crop plants;
2. Identification of marker-trait associations using relevant DH populations (with parents of source populations showing significant phenotypic contrast), further leading to potential use of markers in marker-assisted selection (MAS);
3. High-density genotyping of the DH lines for selection of parental lines with complementary genotypes (or haplotypes) in generating hybrids for further testing;
4. Combining seed-chipping technology in MAS of DH lines for relatively simply inherited traits (e.g., provitamin-A enrichment) using reliable markers for favorable genes/alleles with high contribution to phenotypic variation, which could be cheaper, faster, and more effective than phenotyping the DH lines;
5. Potential usefulness of DH lines in implementing genome-wide selection (or genomic selection or GS; Meuwissen et al., 2001; Jannink et al., 2010) for improving complex polygenic traits with low heritability (e.g., grain yield (GY), abiotic stress tolerance), and when *N* (population size) is small (Bernardo and Yu, 2007; Lorenzana and Bernardo, 2009; Mayor and Bernardo, 2009); and

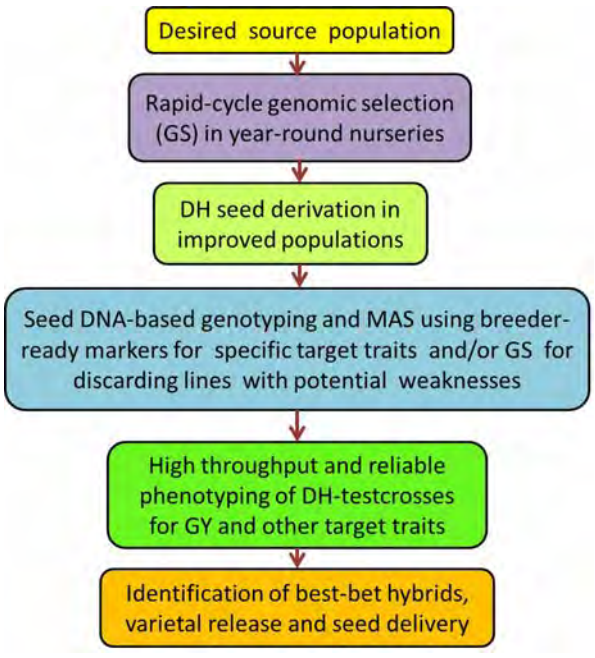


**Figure 2.** An illustrative scheme for enhancing breeding efficiency and genetic gains through a combination of modern technologies/strategies in maize breeding.

6. Potential complementary of DH and MAS for deriving DH lines from bi-parental crosses when the objective is to obtain lines genetically similar to either parent of the cross (Smith et al., 2008) or to identify recombinants at or flanking specific loci. The most frequent application of this approach would likely be the use of DH line conversion protocols instead of slower conventional backcrosses (Forster and Thomas, 2005).

**Future Perspective**

The DH technology, undoubtedly, provides powerful means to modernize the maize breeding operations through simplified logistics and significantly lesser investment of resources for deriving completely homozygous lines for hybrid development and deployment. Implementation of DH technology requires new skills on the part of breeding programs, for both DH line production and integrating DH lines efficiently in the breeding pipeline. Firstly, the major steps in DH line production (haploid induction, haploid identification, chromosome doubling, and DH line recovery) require implementation of effective (and safe) operational practices, and proper training of the concerned scientific/technical personnel. Secondly, the haploid maize plants derived through *in vivo* induction and chromosome doubling are often weak and vulnerable to various environmental stresses, including excessive heat, insect pests, and diseases. Thirdly, the power of DH technology in enhancing genetic gains and breeding efficiency, and ultimately for fast-track development of elite hybrids, can be realized when it is effectively combined with MAS and year-round nurseries. Therefore, to be able to effectively scale-up DH development by institutions based in the tropical/subtropical maize-growing countries, these factors need to be carefully considered.

With financial support from the Bill & Melinda Gates Foundation, CIMMYT will soon be establishing a centralized maize DH facility for sub-Saharan Africa. The facility is expected to serve primarily the DH requirements of public (not-for-profit) research institutions in CIMMYT- and IITA (International Institute of Tropical Agriculture)-led breeding networks, and to provide (over a period of time) low-cost DH service to SME seed companies in the region. CIMMYT also plans to operationalize a DH service facility in Latin America, followed by a similar facility in Asia, through the International Maize Improvement Consortium.

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# 2. *In vivo* Maternal Haploid Induction in Maize

***Vijay Chaikam***

### *In vivo* versus *in vitro* haploid induction

Haploids in maize can be obtained either through *in vitro* (androgenesis) or *in vivo* methods. Androgenesis refers to the development of haploid plants from immature pollen either by anther culture or microspore culture. In anther culture systems, microspores within the anther are induced to undergo androgenesis to form microspore-derived embryo-like structures. In pollen culture, microspores are isolated from anthers and cultured on a medium to produce embryo-like structures. Embryo-like structures can either directly regenerate into haploid plants or indirectly regenerate via the formation of regenerable calli. As microspores are produced in abundance in plant anthers, they are relatively easy to access and manipulate in cultures. Although androgenesis protocols are well established and routinely used in some crop species, obtaining haploids and doubled haploids (DH) through androgenesis has not proved to be efficient in maize. Androgenesis in maize was found to be highly genotype-dependent; most maize genotypes are recalcitrant and do not show any response in culture (Brettell et al., 1981; Genovesi and Collins, 1982; Miao et al., 1981; Spitkó et al., 2006). Even in genotypes that respond to androgenesis, this process is highly influenced by many conditions, including anther stage, donor plant, and anther pretreatment. (Wan et al., 1991; Chu et al., 1975; Ku et al., 1978; Genovesi and Collins, 1982; Miao et al., 1978; Spitkó et al., 2006). Therefore, *in vitro* approaches for DH development are not very commonly used in maize.

In contrast, *in vivo* haploid induction has been highly successful in maize and is now extensively followed by several commercial breeding programs (as discussed in chapter 1 in this manual). Haploids were reported to occur naturally in maize plantings at a frequency of about 0.1% (Chase, 1951). Such a frequency of induction cannot be exploited efficiently for large-scale DH operations. The discovery of Stock 6 (Coe, 1959) and further derivation of an array of maternal haploid inducers in maize, as described earlier in this manual, revolutionized the application of DH technology in maize breeding, as this method is much less dependent on the donor genotypes (source germplasm) from which DH lines are derived.

### Maternal versus paternal haploids

The induction of paternal (androgenetic) haploids is based on a mutant gene, *ig1* (*indeterminate gametophyte*), which can increase the frequency of haploids in its progeny (Kermicle, 1969, 1971; Lin, 1981). Homozygous *ig1* mutants show several embryological abnormalities including egg cells without a nucleus. After fusion with one of the two paternal sperm cells, such an egg cell may develop into a haploid embryo possessing the maternal cytoplasm and only paternal chromosomes. In selected genetic backgrounds, the haploid induction rate ranges from 1% to 2% (Kermicle, 1994).

To produce paternal haploids, the inducer (with *ig1*) is used as the female parent and the donor (source germplasm) as the male parent. Hence, paternal haploids contain the cytoplasm of the inducer and chromosomes from the donor plant. Low frequency of haploids and changes in the constitution of cytoplasm from the donor genotype make this system not very attractive to derive inbred lines for breeding. However, the *ig1*/*ig1* genetic stock can be useful for the conversion of an inbred line to its cytoplasmic male sterile form. The DH plants obtained in this method are isogenic with the male parent except that they carry male-sterile cytoplasm. Inducer lines with various Cytoplasmic Male Sterile (CMS)-inducing cytoplasms have been created, which can be used to transfer new breeding lines into the CMS cytoplasm (Pollacsek, 1992; Schneerman et al., 2000).

For producing maternal haploids, the haploid inducer is used as the male parent in induction crosses, with the source germplasm or donor as the female parent. Maternal haploids carry both cytoplasm and chromosomes from the donor. Many haploid inducer lines with commercially usable and higher haploid induction rates (HIR) are now available, the details of which were provided in the introductory chapter of this manual.

To develop improved haploid inducers adapted to tropical conditions, segregating populations were developed at CIMMYT from crosses between temperate inducers (RWS, UH400, and RWS x RWK- with HIR of 8–10%) and three tropical maize lines developed by CIMMYT (CML494, CML451, and CL02450). A pedigree breeding scheme was followed with mass selection for highly heritable and visually scorable traits on individual F2 plants and family-based selection for HIR and other agronomic characteristics in advanced selfing and backcross generations (Prigge et al., 2011). Tropically adapted inducer lines so developed combined high HIR (ranging from 6% to 13%) with improved pollen production, disease resistance, and plant vigor compared to the temperate inducers under tropical conditions.

**Mechanism of *in vivo maternal* haploid induction**

The exact sequence of events underlying maternal haploid induction has not been clearly understood. Several hypotheses were proposed to explain *in vivo* maternal haploid induction. As haploid induction is achieved when an inducer line is used as a pollen parent, hypotheses were proposed that the regular double fertilization is distorted after pollination with the pollen of a haploid inducer line. In normal double fertilization, one of the two sperm cells from the pollen grain fertilizes the egg cell to form a diploid zygote and the other sperm cell fertilizes the two polar nuclei of the central cell in the female gametophyte, which ultimately develops into triploid endosperm. Pollen from haploid inducers was proposed to cause a distortion in double fertilization in such a way that one sperm cell fuses with the central cell but the other sperm cell does not fuse with the egg cell. But a fertilized and dividing central cell stimulates the unfertilized haploid egg cell to develop into a haploid embryo (Chase, 1969). Such single fertilization could be a result of morphological defects in pollen grains or existence of only a single normal sperm in a pollen grain. Pogna and Marzetti (1977) germinated pollen grains from inducers and non-inducers *in vitro* and observed that pollen grains from inducers exhibited two pollen tubes at high frequency. They proposed that such an abnormality in pollen tube growth may be related to haploid induction capability.

Bylich and Chalyk (1996) noticed about 6.3% of pollen grains with a pair of morphologically different sperm nuclei in haploid inducer line ZMS. They proposed that the morphological differences could possibly arise as two sperms cells develop at different speeds, which could lead to development of one sperm that is in a state ready for fertilization and another that is not. High heterofertilization frequency was noticed with Stock 6 (Sarkar and Coe 1966, 1971). Similar observations were made with inducer line MHI by Rotarenko and Eder (2003). Heterofertilization, usually caused by delayed fertilization, is proposed to be related to the mechanism of haploid induction as well as the HIR. Mahendru and Sarkar (2000), however, could not find any difference between the two sperms in pollen of a haploid inducing line. Swapna and Sarkar (2011) also could not find any defects in pollen tube growth and did not observe delayed fertilization. They proposed attenuation of sperm nuclei after the release from the synergid into the embryo sac as a possible cause of haploid induction.

Chalyk et al. (2003) found 10% to 15% aneuploid microsporocytes in the haploid induction lines MHI and M471H. They proposed that in the haploid inducers, abnormal division of chromosomes occurs during microsporocyte formation, which may lead to development of aneuploid sperm. Aneuploid gametes can break doubled fertilization and stimulate egg cell development into embryo without fertilization.

In contrast to the above, some researchers (Wedzony et al., 2002) indicated that during maternal haploid induction, normal fertilization might still occur, but during the subsequent cell divisions, the inducer chromosomes degenerate and are then eliminated from the primordial cells. Fischer (2004) used microsatellite markers to check for strictly maternal origin of haploids induced by RWS. About 1.4% of the genotypes possessed one or, rarely, several inducer chromosome segments. Generally, these segments had replaced the homologous maternal segments. Li et al. (2009) and Zhang et al. (2008) demonstrated that chromosomal segments from inducer parent are integrated into the genome of the haploids and doubled haploids, suggesting elimination of chromosomes from the inducer parent after fertilization.

Taking all of this information together, the mechanism of haploid induction is yet to be conclusively elucidated. However, it is certain that some reproductive abnormalities are involved, and it is also possible that different inducers may cause different reproductive abnormalities leading to maternal haploid formation.

**Genetics and molecular marker analysis of maternal haploid induction**

Studies on segregating generations derived from crosses between inducer and non-inducer parents revealed continuous variation for haploid induction associated traits and indicated that the *in vivo* haploid induction trait is under polygenic control (Lashermes and Beckert, 1988; Deimling et al., 1997; Röber et al., 2005, Vanessa et al., 2011). Lashermes and Beckert (1988) inferred that the haploid induction trait of the Stock 6 inducer line is a dominant character with nuclear determination and is controlled by a few major genes.

Deimling et al. (1997) and Röber (1999) used Restriction Fragment Length Polymorphism (RFLP) markers and identified two QTL (on chromosomes 1 and 2) responsible for haploid induction in an F3 population involving Stock 6 and W23*ig* as parents. These QTL together explained 17.9% of the phenotypic variance and 40.7% of the genotypic variance in haploid induction rates. The positive QTL allele on chromosome 1 was dominant and originated from Stock 6 whereas the one on chromosome 2 was additive and originated from W23*ig.*

In another study, Barret et al. (2008) found segregation distortion in a population developed from a cross between a non-inducer and an inducer line (PK6). This analysis revealed a major locus on chromosome 1 covering 11.6 cM in bin 1.04 for haploid induction. Fine mapping based on synteny with rice chromosomes led to identification of two Sequence-Tagged Site (STS) markers closely linked to the induction locus (4.5 and 4.9 cM, respectively). This fine-mapped region contained 28 putative expressed genes.

Prigge et al. (2012) conducted comparative QTL mapping involving four segregating mapping populations, which were developed by crossing haploid inducer line UH400 with two temperate (CAUHOI, 1680) and two tropical (CML395, CML495) inbreds. In three of these populations a major QTL was identified for haploid induction on chromosome 1 (bin 1.04) explaining up to 66% of the genetic variance. The loci in bin 1.04 exhibited segregation distortion against the UH400 allele in these three populations. In another segregating population involving two inducer lines as parents (CAUHOI × UH400), seven QTL were identified on five chromosomes, with one QTL on chromosome 9 contributing 20% in three generations of this cross. The results led to the suggestion of pyramiding of major QTL on chromosome 1 and minor QTLs could lead to further improvement in induction capabilities.

**Source germplasm for haploid induction**

The choice of source germplasm or donor for haploid induction depends on the objectives of the breeding programs. Usually breeders induce haploids on the F1 or F2 populations. It was estimated that an F2-derived DH may contain almost 50% more of the best recombinants than an F1-derived population (Gallais, 1990). However, the difference in the frequency of the best recombinants between F2- and F3-derived populations is small. This implies that the DH approach is better followed on F2 populations when linkage is observed between genes (Gallais, 1990; Bernardo, 2009).

In maize, a high mutational load of deleterious recessive alleles hampers exploiting the genetic potential of allogamous landraces in hybrid breeding. It was proposed that the DH technology could be an effective approach for eliminating deleterious recessives from a gene pool (Gallais, 1990, Wilde et al., 2010). Even though landrace-derived lines may not be directly used as parents in hybrid breeding programs because of significant differences in performance for agronomically important traits as compared to elite inbred lines, they may be valuable genetic resources for marker-assisted backcrossing or pre-breeding activities (Wilde et al., 2010). Compared to elite inbred lines, landrace-derived DH lines are much closer to Hardy-Weinberg equilibrium, which allow detection and mapping of QTL with high accuracy and resolution. So land race derived DH lines are ideally suited for marker-trait association studies (Wilde et al., 2010).

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# 3. Design and Implementation of Maternal Haploid Induction

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For successfully producing an optimal number of doubled haploid (DH) lines from a source population, the first critical step is to produce enough haploid seeds from the induction crosses. This will depend on three important factors: (1) haploid induction rate (HIR) and pollen production capabilities of inducer used, (2) total number of successful induction crosses, and (3) lack of anthocyanin color inhibitors in the source population. The design of the induction nursery also affects the efficiency in handling the pollinations and the number of successful induction crosses. These factors need to be thoroughly considered before planting the induction nursery.

**Selection of inducer lines for haploid induction**

Inducer lines for the haploid induction nursery should be selected based on HIR, pollen production, plant height, vigor and per se performance of the inducer, flowering behavior, resistance to diseases and insects, and ease of maintenance of the inducer in the target environment. For large-scale commercial application of DH technology, haploid inducers with high HIR should be chosen for the induction nursery. As mentioned in chapter 1, several inducer lines have been developed with an average HIR above 6%. However, most of these inducer lines are better adapted to temperate environments. CIMMYT has been using temperate inducer lines UH400, RWS, and their hybrid for haploid induction in tropical and subtropical environments in Mexico. The HIR of these temperate inducers is maintained at similar levels (~8–10%) in tropical and subtropical environments. However, these temperate inducer lines and their hybrids exhibit poor agronomic characteristics and disease vulnerability in tropical environments. Nevertheless, it is possible to obtain pollen for haploid induction with multiple sprays of fungicides, insecticides, foliar nutrition, and other best agronomic management practices. Temperate inducer lines and their hybrids are very short in height, which makes them almost impossible to use in isolation blocks with open pollinations, thereby necessitating expensive manual pollinations. Extremely early flowering and a very brief period of pollen shedding also make it necessary to stagger inducer lines multiple times to coincide flowering with tropical source germplasm. Seed production and maintenance of temperate inducers are also problems in tropical conditions as they produce very small ears which are susceptible to ear rots. Temperate inducers show comparatively better performance in winter induction nurseries than summer nurseries in tropical environments in Mexico.

In contrast, the tropicalized haploid inducer lines (TAILs) developed at CIMMYT-Mexico, in collaboration with the University of Hohenheim, exhibit better agronomic characteristics in terms of flowering and pollen production in tropical environments, while maintaining high HIR of 8–12%.The TAILs also exhibit better resistance to tropical diseases and insects, making agronomic management less expensive in tropical environments. Hybrids of tropical inducers are taller compared to temperate inducer hybrids, so they can be used in an isolation nursery with open pollination. Seed production and line management are also comparatively easy for TAILs in tropical environments.


**Figure 1**. Temperate and tropical inducer lines at CIMMYT El Batán experimental station, Mexico.

**Number of induction crosses**

The number of induction crosses per source population depends on the number of haploid seeds to be produced per source population, which in turn depends on the target number of DH lines to be produced. At CIMMYT, we aim to produce 200 DH lines from each source population. At a 10% success rate in chromosomal doubling we need at least 2,000 haploid seeds to obtain 200 DH lines. At an 8% induction rate and 200 kernels per ear we need to have 125 successful crosses to obtain 2,000 haploids. We plant at least 150 plants to allow for plant losses due to non-germination and post-germination death of the plants.

**Manual vs. open pollination in the haploid induction nursery**

Induction crosses can be conducted in an isolation nursery using open pollination or could be conducted in a nursery using manual pollinations. The decision to use open pollination or manual pollination depends on several factors.

An isolation nursery with open pollinations can be a chosen when:

- It is possible to plant an induction nursery at least one month earlier than the rest of the maize plantings in the surrounding area;
- The source populations do not differ in their silk emergence date by more than 15-20 days;
- There are more than 50 source populations; and
- A taller inducer or inducer hybrid is available that reaches at least the height of the ears of the source population plants.

Manual pollinations may be preferred in an induction nursery when:

- Few populations to be induced;
- When flowering time information is not available for source populations
- Source populations have a wide range of maturity;
- Isolation by early planting would not be possible; and
- The inducers used are very short in height relative to the source populations.

**Design of the induction nursery**

A good design of the induction nursery is important for efficient handling of pollinations and to achieve success in haploid induction. The same field design can be used for the induction nursery with isolation using open pollinations and an induction nursery using manual pollinations. Flowering time information for the source population (days to silking) and inducers (days to anthesis) is necessary for designing the induction nursery. All source populations with similar silking time can be grouped and planted in the same area of the nursery so that pollinations can be handled easily.

At CIMMYT's DH nursery in the Agua Fría experimental station, seeds from the source populations are sown in 4.5 m long rows at a spacing of 25 cm. Each row accommodates 19 plants. The spacing between rows is maintained at 75 cm. Haploid inducer lines are planted in long ranges. A typical design for an induction nursery is represented in Figures 2 and 3. Since the tropical and temperate inducer lines flower much earlier than the tropical source germplasm, planting of source populations can be done earlier. All the source populations with early to intermediate silking dates can be accommodated in the front to the middle of the nursery. Other populations with intermediate to late silking dates can be accommodated from the middle to the end of the nursery. For each source population, eight rows are planted. After every four rows of source populations, two long ranges (highlighted in yellow in Figure 3) are left for inducer planting. Also, two horizontal ranges in the front and two horizontal ranges in the back (highlighted in yellow in Figure 3) of the induction nursery are left for inducer planting. Inducer plantings need to be staggered at weekly intervals depending on the variability in silking dates of source populations. The first inducer planting is done one week after planting the source population in the first vertical long ranges. The second inducer planting is done 14 days after planting the source population in the second vertical long ranges. The third planting of inducers is done in the front two ranges 21 days after planting the source population. The fourth inducer planting is done in one or two of the horizontal ranges at the end of the induction nursery 28 days after planting the source populations. If needed, a fifth inducer planting can be done in the second horizontal range after another week. In this design, 150 source populations can be accommodated per hectare along with necessary numbers of inducer plants.

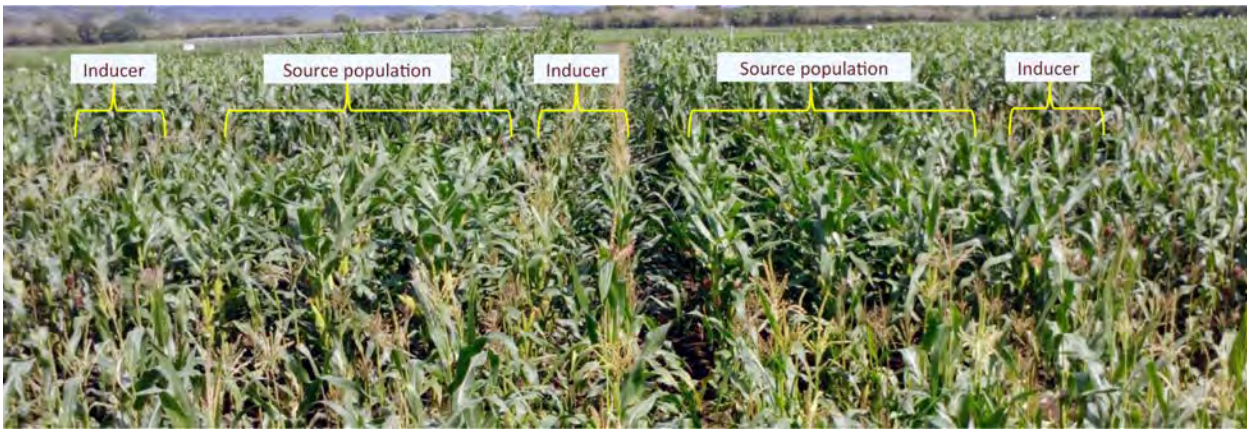
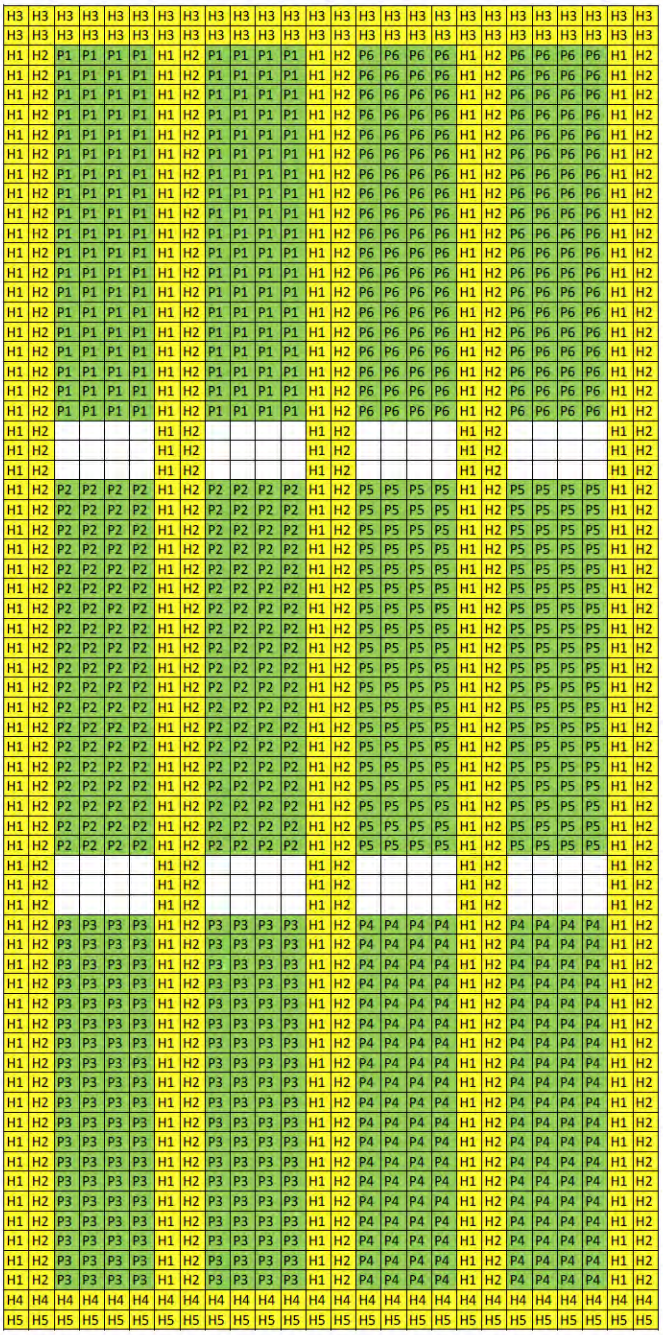



**Figure 2.** Haploid induction nursery in CIMMYT Agua Fría experimental station, Mexico.



H1, H2, H3, H4, and H5: First, second, third, fourth, and fifth plantings of the haploid inducer, respectively
P1, P6: Early maturing source populations
P2, P5: Medium maturity source populations
P3, P4: Late maturing source populations

**Figure 3.** Typical design of a haploid induction nursery at CIMMYT.

**Management of induction nursery**

Since the isolation block is planted very early compared to other maize plantings, seeds and seedlings may be prone to fungal or insect attacks depending on the environment and local biotic stress pressure. In such cases, seed treatment will aid in combating fungal pathogens and insects during germination and early seedling stages. Seeds may be treated with a mixture of fungicides and insecticides. Gaucho, a systemic insecticide used for seed treatment, is effective for combating insect attack during seedling stages. During soil preparation, fertilizer (75-80-60 NPK/ha), pre-emergent herbicide (Atrazine), and insecticide (Lorsban 5G) are incorporated into the soil. Before planting, plots are irrigated. After germination, plots are irrigated based on the soil conditions. A second application of fertilizer (150-80-

60 NPK/ha) may be done after 40 days. Paraquat may be applied when plants are 40 to 50 days old for managing the weeds.

In the induction nursery, inducers need to be given special care, as they could be weak and vulnerable to diseases and insects. In such cases, the inducer plants may be sprayed with fungicides and insecticides. Turcicum leaf blight, rust, tar spot complex, *Bipolaris maydis,* and ear rots are common diseases affecting maize in the tropical environments of Mexico. These diseases can be effectively controlled by application of Tilt (Propicanazole-0.5L/ha) at 15-day intervals.

Armyworm *(Spodoptera frugiperda)* larvae may also cause damage in the induction nursery, depending on the local conditions. This can be controlled by the insecticide Palgus (Spinetoram) during the seedling stage and Larsbon 3G (Chlorpyrifos ethyl) during later stages of plant growth. Karate Zeon (Lambda Cyalotrina) can also be applied to control armyworm. When severe infection of armyworm occurs, a mixture of Larsbon, Karate Zeon, and Palgus is applied. In some environments/locations, greenhoppers, which are carriers for corn stunt complex (Spiroplasma, Phytoplsama, Raillophena), need to be managed in early plantings. During ear development, *Spodoptera litura* may cause considerable damage, which needs to be effectively managed.

**Pollinations in the induction nursery**

For both manual and open pollinations, detasseling, i.e., removal of the tassels from source populations immediately after they appear (to minimize pollen contamination), is done. In an isolation nursery with open pollinations, the ears should not be covered with shoot bags. Open pollinations can be aided by dispersing the pollen using a hand blower.

In an induction nursery with manual pollinations, ears should be covered by shoot bags before silking occurs. Ear shoot may be neatly cut at the tip one day before conducting manual pollinations to aid uniform growth of silks. Pollen from ~10 inducer parents is bulked and adequately applied on the silks. Each ear may be pollinated twice, if needed, on two consecutive days to obtain ears with good/complete seed sets. During development and drying, the ears need to be properly protected from birds.

**Handling of the harvested ears**

Ears from each of the source populations in an induction nursery can be harvested independent of other source populations when all the ears in that population reach physiological maturity. This prevents losses due to ear damage by pathogens and insect pests. All the ears from same population should be harvested in one or two bigger mesh bags that are clearly labeled. Harvested ears are dipped briefly in the insecticide deltametrina (125 ml/200 lts water) to control insect pests and are then dried completely in sunlight for two to three days. Once the ears are dried, they are shelled and seed is collected in a labeled mesh bag and kept in a cold storage room until ready for sorting.

> ***Note***: Mention of specific brand names of commercial chemicals (including fertilizers, fungicides, and pesticides) is not intended as an official endorsement of the product by CIMMYT. There may be other equal or better products available in the market for achieving the same task.

**HIR assessment of the haploid inducers**

Assessment of the haploid inducer lines for HIR requires suitable testers that allow unambiguous identification of haploids at the early seedling or kernel stage. Most commonly used testers possess recessive genes like *liguleless* and *glossy*. When the testcross kernels from the cross *liguleless* × inducer or *glossy* × inducer are germinated, only haploid seedlings exhibit the glossy or liguleless phenotypes. These assays can be conducted on seedlings (at the three-to four-leaf stage) in a greenhouse.

It is also possible to use an *R1-nj* anthocyanin marker system for assessment of HIR in inducer lines that incorporate *R1-nj*. For this system to be effective, testers should be identified that do not possess inhibitor genes and that express *R1-nj* very well under different environments. Inducers can be crossed to such testers and HIR determined based on *R1-nj* expression.

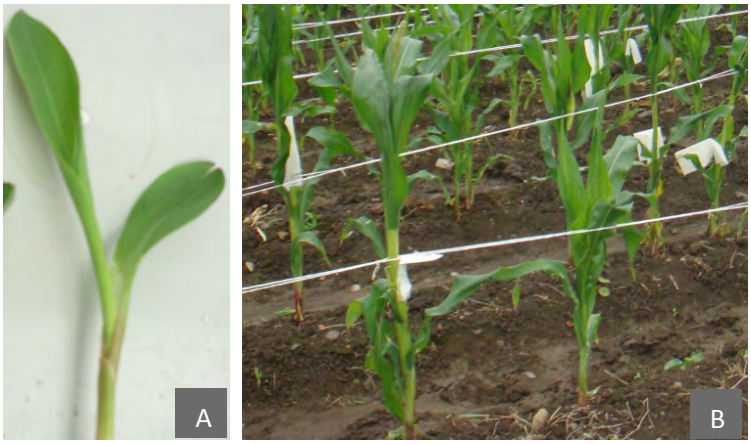



**Figure 4**. *Liguleless* phenotype in the (A) seedling and (B) adult plant stages. The *liguleless* phenotype is characterized by lack of the ligule and the auricle, and by erect leaves.

**Maintenance breeding of the haploid inducers**

A continuous selection and testing system should be established for maintaining the high haploid induction rate as the genetic factors controlling haploid induction undergo segregation distortion and are selected against by the nature. In maintenance breeding of the inducer lines, the greatest emphasis should be given to retaining the high haploid induction rate and maintaining the anthocyanin marker system. Importance should also be given to pollen production characteristics and vigor of the plants. Sib-mating is recommended rather than selfing to maintain the vigor of the inducer.

Inducer plants in the seed multiplication plot are scored for various agronomic and phenotypic traits (e.g., pollen production ability, plant height, vigor, expression of purple color on the stem, disease resistance, and ear traits). Plants with the best *per se* performance are selected and tagged. Pollen is collected from selected plants in a row and bulked, and the bulked pollen is used for sib-mating (within the same row) and for testcross to a recessive tester suitable for HIR assessment. All the ears harvested from a row are shelled and kept separately.

Testcross seeds from each row of the inducer multiplication plot are planted separately in a greenhouse. Seedlings are evaluated for the recessive trait at the three- to four-leaf stage and HIR is assessed. Inducer rows with high HIR are identified, and seeds from each ear of that row are scored for intensity and proper expression of purple coloration on the embryo and endosperm. Ears from plants with the best agronomic scores and with good expression of color marker on the embryo and endosperm are selected. These ears are used for maintaining the inducer stocks.

# 4. Maternal Haploid Detection using Anthocyanin Markers

*Vijay Chaikam and BM Prasanna*

**Introduction**

Haploid plants can be distinguished from diploid plants by characteristics like erect leaves, poor vigor, and sterility. These characteristics can only be observed after sufficient growth of haploid plants. Distinguishing haploids from diploids at seed level offers many advantages like saving costs involved in artificial chromosomal doubling and saving greenhouse and field space and labor. So identification of haploids at seed level is critical for adapting DH technology on a commercial scale. A commercially usable, ingenious phenotypic marker system based on anthocyanin coloration was identified in the 1960s (Nanda and Chase, 1966; Greenblatt and Bock, 1967) to distinguish haploids from diploid at the seed stage. Integration of anthocyanin markers in haploid inducer lines facilitated haploid identification not only at the seed level but also at different stages of plant growth.

**Identification of haploid kernels using an *R1-nj* marker system**

*R1-nj (R1-Navajo),* a dominant variant allele of the *R1* locus, is now widely used for the screening of haploids in kernels. *R1-nj* in combination with other dominant genes in the anthocyanin synthesis pathway (*A1, A2, Bz1, Bz2, C1, and C2*) causes deep pigmentation of the aleurone (endosperm tissue) in the crown (top) region of the kernel (Coe, 1994). In addition, it conditions purple pigmentation in the scutellum (embryo tissue). This phenotype is called the Navajo kernel phenotype.

In haploid inducer lines that are commonly used now, the *R1-nj* allele is integrated along with other genes necessary for anthocyanin biosynthesis. Most of the maize germplasm used in breeding programs does not have *R1-nj* allele or anthocyanin biosynthetic genes that confer purple/red pigmentation in the kernel/plant tissues. When the inducer lines are crossed (as male parent) to the source germplasm (as female parent) not having the anthocyanin color markers, all the resulting hybrid kernels are expected to express the Navajo phenotype in the endosperm and in the scutellum (embryo) as *R1-nj* is dominant over the colorless *r1* allele. Thus, the differential expression of *R1-nj* facilitates identification of maternal haploids from the diploid kernels. When haploid inducers with a high haploid induction rate (HIR) are used in the induction cross, maternal haploids usually occur at a frequency of 6–10%.

In practice, three types of kernels may be obtained from the induction cross:

(1) Normal diploid or hybrid kernels with purple coloration on the endosperm (aleurone) and the embryo (scutellum);
(2) Haploid kernels with purple endosperm but no coloration on the embryo; and
(3) Kernels without purple coloration on the embryo and endosperm, which could be due to pollen contamination.

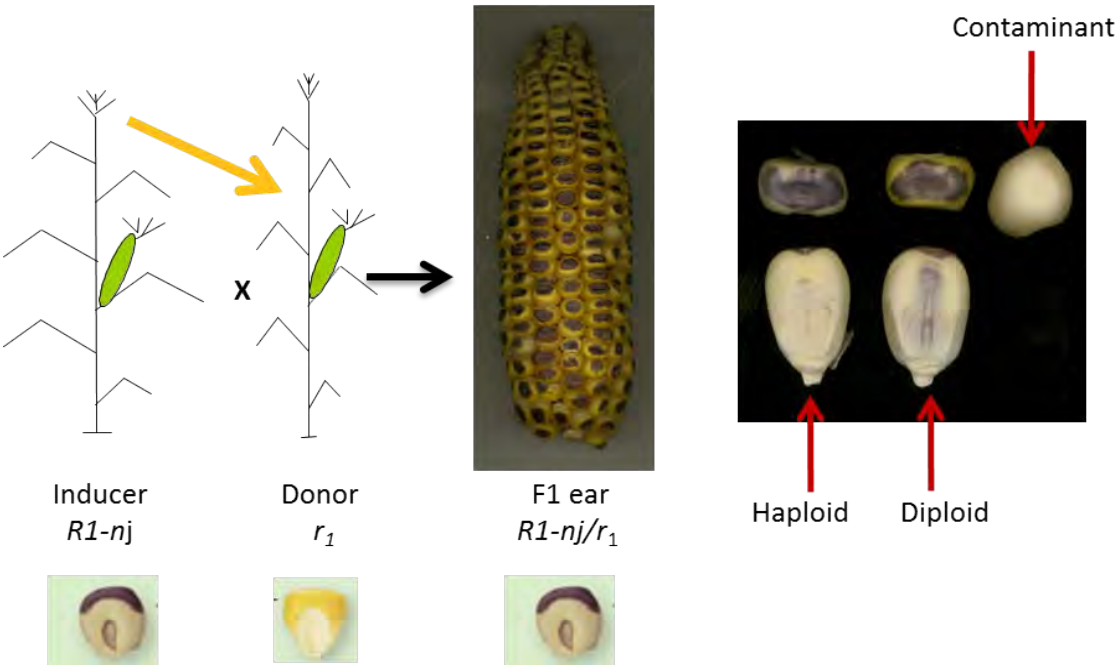



**Figure 1.** Illustration of maternal haploid induction and kernel types obtained through a typical induction cross.

Even though the *R1-nj* marker system offers an efficient way to identify haploids, *R1-nj* expression could be highly influenced by the genetic background of the female parent. The Navajo crown pigmentation might vary from a small spot (at the silk attachment region of the kernel) to covering the entire aleurone (except the base). Also, the intensity of color on the aleurone may vary from very pale to deep. Expression of color on the scutellum may also vary from pale to deep (Figure 2).

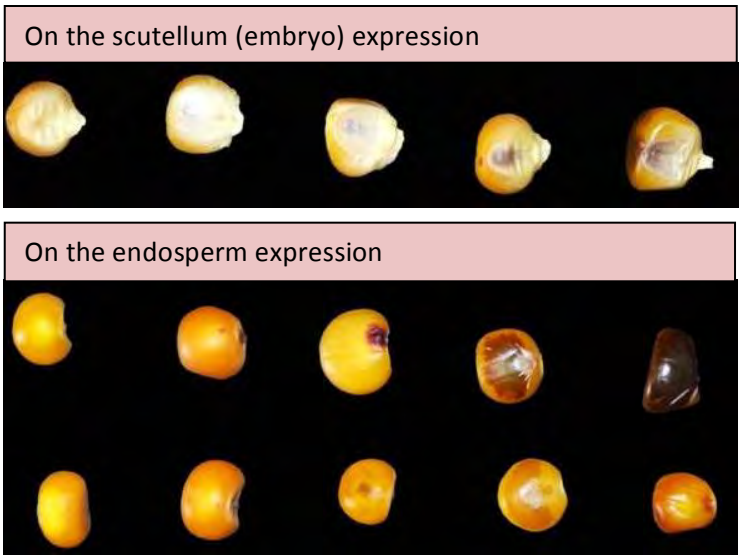



**Figure 2.** Variation in *R1-nj* expression on the embryo and endosperm.

The variation in *R1-nj* expression can lead to different outcomes as follows:

(1) Whole endosperm and all the embryo tissues become colored: haploid identification is easy.
(2) Good coloration on the crown of the endosperm and scutellum: haploid identification is easy.

(3) Only a purple spot on the crown of the endosperm and slight expression on the embryo: haploid identification is possible, but high false positives could happen due to difficulties in haploid identification.
(4) Completely inhibited on both endosperm and embryo: impossible to identify haploids.
(5) Completely inhibited on the endosperm but embryo tissue is marked to some extent: in such cases, all the kernels with colored embryos can be considered diploid. But it is not possible to distinguish haploids and pollen contaminants from this category.

**Limitations of *R1-nj* system**

(1) When the source populations contain dominant anthocyanin inhibitor genes such as *C1-I,* which are common in flint maize (Röber et al., 2005), *R1-nj* color marker expression is completely suppressed and haploid kernel identification is almost impossible. CIMMYT's elite germplasm is currently being surveyed to determine in what proportion the seed color marker will function, permitting efficient haploid seed detection. Currently, it appears that *R1-nj* color expression is inhibited in only about 8% of crosses of haploid inducers with diverse source populations.
(2) When F1 or F2 populations are used as source materials and if only one parent has an inhibitor gene, kernels will be segregating for Navajo phenotype. In such cases, one may not be able to identify all the haploid kernels efficiently and could potentially lose half to three-fourths of the haploids.
(3) The accuracy and speed of haploid identification depends on trained staff with good understanding of haploid detection through the color expression on endosperm and embryo.
(4) Automation of haploid identification is difficult, if not impossible, using this system.
(5) Moisture of kernels at the time of harvest could potentially affect the intensity of color expression (Rotarenco et al., 2010).

**Purple root and purple stem markers for haploid identification**

In view of the above-mentioned limitations of the *R1-nj* color marker system in haploid detection, some researchers have explored the possibility of additional color markers, especially those expressed in root and stem, for reliable identification of maternal haploids (Rotarenco et al., 2010). Two such genes that can impart purple or red color to the plant tissues are *Pl1 (Purple1)*, which conditions sunlight-independent purple pigmentation in plant tissues, and *B1 (Booster1)*, which conditions sunlight-dependent purple pigmentation in most of the above-ground plant tissues (Coe, 1994).

The *B1* and *Pl1* genes can be integrated into the inducer lines along with the *R1-nj* marker system. When Navajo coloration is not expressed on the kernels, haploids can be identified based on the seedling root color or stem color in the field. When such an inducer is crossed with source material, diploid plants will have purple roots and stems, while the putative doubled haploid plants will not express such coloration.

Some temperate inducer lines like MHI (Eder and Chalyk, 2002) and Procera Haploid Inducer (Rotarenco et al., 2010) combine *R1-nj* with *B1* and *Pl1* genes for more effective identification of haploids.

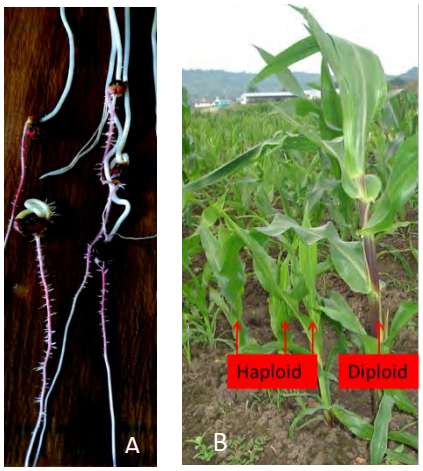



**Figure 3**. (A) Purple color expression in the roots; (B) purple stem color in diploid plants and normal green stem in putative doubled haploid plants.

**Limitations of *B1* and *Pl1* system**:

(1) Many source materials contain *B1* and *Pl1* genes. In such source populations, haploid plants also express coloration in the root and stem, making it almost impossible to reliably identify haploid plants.

(2) Expression of the *B1* and *Pl1* genes are affected by plant growth conditions, especially sunlight and temperature. It was observed that purple pigments accumulate best under low temperatures.

**Further possibilities**

Some research teams are exploring novel marker systems that can potentially facilitate automated haploid detection with minimal false positives. Rotarenco et al. (2007) proposed haploid identification based on kernel oil content, determination of which can be potentially automated using nuclear magnetic resonance–based techniques. Li et al. (2009) recently developed CAUHOI, a Stock6-derived inducer (with ~2% HIR and high kernel oil content (78 g $kg^{-1}$)) that allows identification of haploids based on both lack of *R1-nj* conferred scutellum coloration and low embryo oil content. This novel approach looks promising, but its reliability and applicability for high-throughput DH production in tropical genetic backgrounds remains to be investigated.

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# 5. Chromosome Doubling of Maternal Haploids

*Vijay Chaikam and George Mahuku*

**Introduction**

Diploid plants contain two copies of each chromosome in their cells, of which one copy is received from the male parent and the other from the female parent. In the reproductive structures (tassel and ear in maize), haploid male (pollen grain) and female (embryo sac) gametophytes are results of meiotic cell divisions which involve pairing of homologous chromosomes and recombination.

Haploid plants contain only one copy of each chromosome in their cells. Haploids derived by maternal *in vivo* induction contain chromosomes only from the female parent. In the reproductive structures of haploid plants, meiotic cell divisions cannot proceed as homologous chromosomal pairs cannot form, resulting in non-production of male and female gametophytes and gametic cells. So haploid plants are usually sterile. The purpose of chromosomal doubling is, therefore, to achieve fertility in haploid plants by generating a doubled haploid (2*n*) plant out of a haploid (n), so that these plants can be selfed to derive doubled haploid (DH) lines.

**Mechanism of chromosomal doubling**

Spontaneous chromosomal doubling occurs at low frequency, resulting in fertility of some haploid plants. The frequency of spontaneous doubling is dependent on the genotype of the source population. To achieve consistent and high frequency of chromosomal doubling, haploid plants are treated with chemicals called mitotic inhibitors. These chemicals alter the regular mitosis in such a way that only a single cell with double the number of chromosomes results after mitosis. A commonly used chemical is colchicine, which is a water-soluble alkaloid produced from the bulbs of *Colchicum autumnale*. In the presence of colchicine, replication of chromosomes occurs normally in interphase. Colchicine binds to tubulins and prevents the formation of spindle microtubules during the metaphase stage of mitosis. During anaphase, two sister chromatids in a replicated chromosomes are separate but cannot move to opposite poles of the cell and instead stay at the center of the cell. In telophase, a nuclear membrane is formed around the unseparated chromosomes. So after mitosis a cell with double the number of chromosomes results.

In plants, all the above-ground organs including reproductive structures arise from the shoot apical meristem (SAM). The SAM contains meristematic cells, which divide and differentiate into organ primordia. To achieve complete fertility in reproductive tissues of haploid plants, chromosomal doubling of meristematic cells should occur before they differentiate into reproductive organs. Therefore, exposing very young seedlings (three to five days after sowing) to mitotic inhibitors is recommended.

**Facilities required for chromosomal doubling**

For operational convenience and safety of the workers, chromosomal doubling work can be segregated into three work areas:

(1) A germination room where the seeds are processed and germinated and seedlings are prepared for colchicine treatment. This germination room is equipped with work benches for workers to process the seed and to prepare the seedlings for treatment. This room should also be equipped with an incubator for seed germination.
(2) A colchicine treatment lab where chemicals are stored, colchicine solution is prepared, and seedlings are treated. This room should be equipped with a refrigerator to store chemicals, a fume hood to prepare solutions, colchicine treatment tanks, and an exhaust.
(3) A room where colchicine waste is stored until processed through chemical waste management. This room should be equipped with an exhaust fan.

***Supplies needed for seed germination and seedling processing:***

- Germination paper
- Plastic tubs
- Seed spreaders
- Scalpels and blades

***Supplies needed for colchicine treatment and waste management:***

- Refrigerator
- Weighing balance
- Measuring cylinders: 5,000ml; 1,000ml; 500 ml; 100ml
- Pipettes: 1,000 microliters; 200 microliters; 100 microliters
- Magnetic stirrer with magnets
- Containers to prepare solutions
- Metallic tanks for treatment
- Metallic tanks or polypropylene containers to collect waste
- Protective clothes, gloves, and masks

***Chemical supplies needed:***

- Colchicine
- DMSO
- Bleach

**Steps in chromosome doubling**

***Seed germination*:**

- Germination paper is marked by a cut at one of the corners and moistened with 0.05% bleach solution to prevent fungal growth.
- Two germination papers are spread on top of one another, aligning the cut ends, and seeds are spread evenly using a spreader (Figure 1).
- Seeds are placed with the embryo side facing down and the radicle emergence side placed towards the cut end of the paper (Figure 2). Then seeds are covered with one more paper on the top aligning the cut ends (Figure 3).
- These three layers of germination paper with seeds are folded tightly into a bundle and tied with rubber bands at both ends (Figures 3, 4, and 5).
- Then bundles of seed from the same population are kept in a mesh bag vertically with cut ends facing down and placed in plastic containers with bleach solution (Figure 6 and 7). Bleach solution in the tub helps to prevent fungal growth and maintains humidity.
- Plastic tubs are placed in the incubation chamber (Figure 8), where temperature is maintained around 25 to 28$^{o}$C. Seeds are allowed to germinate for 72 hours.

***Preparation of seedlings:***

- Three days after incubation, the plastic containers with seed bundles are removed from the incubation chamber. The bundles are spread out on a work table (Figures 9 and 10).
- Seedlings with a root length of 3–5 cm and coleoptile length of about 2 cm are ideal for colchicine treatment. Before colchicine treatment, root and shoot tissues are cut at about 2 cm and 1 cm from the tip, respectively, using a sterile blade fixed to a scalpel blade holder (Figure 11). Blades are sterilized by heating them over an alcohol lamp. Cutting the root tips aids in easy handling of seedlings during transplanting, and cutting shoot tips enhances the exposure of the SAM to colchicine treatment.

- Cut seedlings that belong to the same population are kept in a mesh bag (Figure 12).
- Mesh bags with seedlings are kept in water for a few hours before transferring to the colchicine tank (Figure 13).
- The germination paper with very small seedlings and non-germinated seeds can be bundled again and kept in a growth chamber for one more day. The same procedure of seedling cutting can be followed for the next two days.

**Fig. 1.** Spreading of seeds on germination paper

**Fig. 2.** Aligning the seed with the radicle side facing the cut end of the paper, and embryo side facing down

**Fig. 3.** Covering the seeds with a germination paper

**Fig. 4.** Bundling the germination papers with seeds

**Fig. 5.** Bundle formed after rolling

**Fig. 6.** Bundles in a mesh

**Fig. 7.** Bundles kept in a plastic tub with bleach solution

**Fig. 8.** Plastic tubs (with seeds) in an incubator

**Fig. 9.** Opening the bundles

**Fig. 10.** Germinated haploid seedlings

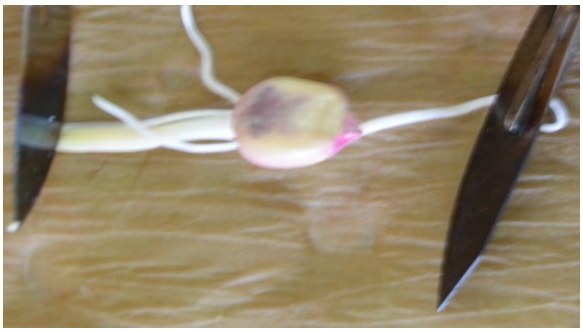

**Fig. 11.** Cutting the root and shoot of germinated seedlings

**Fig. 12.** Cut seedlings in a mesh bag

**Fig. 13.** Mesh bags with seedlings kept in water until treated with colchicine

***Colchicine treatment:***

Since colchicine is very toxic and carcinogenic to human beings, care must be taken to avoid exposure to it by taking necessary precautions. Seedlings can be treated with colchicine in the dark in specialized tanks that allow workers to avoid direct contact with colchicine. These tanks are made up of stainless steel to avoid corrosion (Figure 14). The lid of the tank has an opening, which can be connected to a running water supply or to a container with colchicine solution (Figure 15A). The tank is equipped with an outlet at the bottom center to allow drainage of the spent liquid (Figure 15B). The tank is placed at a height on a stand with iron legs. This permits placement of containers under the tank to collect the waste. The base has wheels for easy movability (Figure 14).

The volume of the colchicine solution required is estimated by placing the cut seedlings in the treatment tank (Figure 16) and pumping water gently until all the seedlings are immersed. Water is emptied into a container from the bottom opening (Figure 15B), and the collected water is measured. This volume represents the amount of colchicine solution that needs to be prepared.

A solution with 0.04% colchicine and 0.5% DMSO is used for chromosomal doubling. Colchicine powder is weighed in a fume hood and dissolved in water in a plastic tank wrapped with aluminum foil. With the aid of a magnetic stirrer, colchicine powder is dissolved in water along with DMSO for two to three hours. The person preparing this solution should wear overalls, gloves, and protective facial cover. The container in which the colchicine solution is prepared has an outlet at the bottom which can be connected to a pipe and an automatic dispenser pump to dispense colchicine solution into the treatment tank automatically. Seedlings are kept in the colchicine tank for 12 hours. For convenience, the treatment can be started at 8 P.M. and stopped at 8 A.M. The spent colchicine solution is collected into plastic containers by opening the outlet at the bottom of the treatment tank. Seedlings are washed at least three times by pumping distilled water into the tank. Waste is collected in big plastic containers and stored in a separate room along with spent colchicine solution until processed by chemical waste management.

**Fig. 14.** Colchicine tank and pumping of colchicine solution from container to the tank


**Fig. 15.** (A) Lid of the colchicine tank with connection to water supply or colchicine container; (B) Collection of colchicine waste from the bottom outlet.

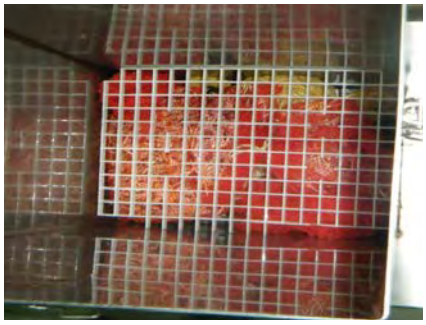

**Fig. 16.** Cut seedlings in mesh bags in colchicine tank

***Seedling transplanting and greenhouse care:***

- Seedlings taken out of the treatment tank are immediately transplanted into Styrofoam trays containing promix (peat moss)(Figure 17,18 and 19). Seedlings should be handled very carefully as they become brittle after colchicine treatments. Seedlings with long hypocotyls are more susceptible to damage.
- Seedlings are maintained in the Styrofoam trays for three weeks in a greenhouse where temperature is maintained at 28–30°C. Seedlings are irrigated gently from the top every evening. For the first irrigation, water is used. From the second irrigation, Hakaphos (13-40-13 NPK and micronutrients) is applied, which helps in root growth and seedling establishment.
- To prevent fungal attacks, the fungicide Tecto (Thiabendazol) is applied every third day. Gaucho (Imidacloprid), which is a systemic insecticide, is applied once a week before transplanting to prevent insect damage. Hakaphos and Gaucho can be combined for application.

**Success rates in different steps of chromosomal doubling**

At CIMMYT a germination percentage of 85–90% is commonly achieved among the putative haploid kernels. During chromosomal doubling, a considerable number of seedlings could be lost due to the toxic effects of colchicine, which is dependent on the genetic background of the source material and the procedure followed for application. Only 40–80% of treated putative haploid seedlings will be established in the field. Among the established plants, 10–30% false positives (diploids) may be noticed, and these should be spotted and removed before flowering. Among the remaining true haploids, 0–40% of plants produce both pollen and silks so that successful pollinations can be conducted. Pollen fertility is again dependent on the genotype of the source germplasm. Only 30–50% of the pollinated plants usually produce seed.

**Fig. 17.** Transplanting of treated seedlings

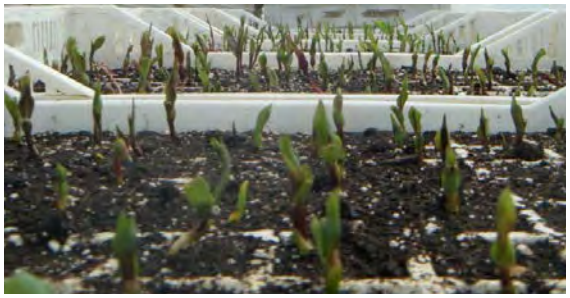

**Fig. 18.** Transplanted seedlings after one week

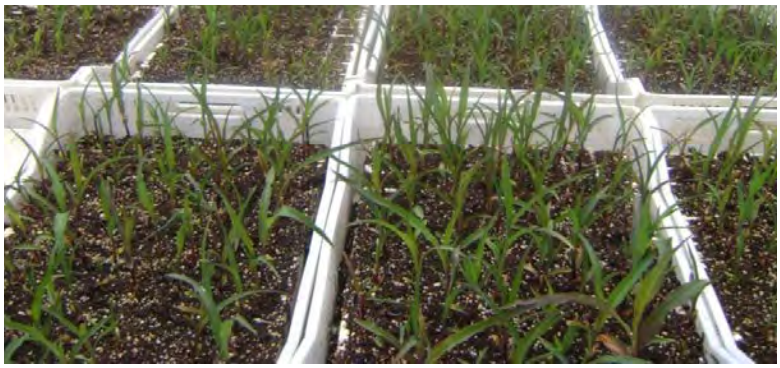

**Fig. 19.** Transplanted seedlings after 3 weeks

**Colchicine toxicity and precautions to be taken**

***Colchicine toxicity:***

At concentrations of 0.1–1 g/ml, colchicine can cause the mitotic arrest of dividing cells (both plant and animal cells) at metaphase by interfering with microtubule organization, in particular those of the mitotic spindle. Colchicine is fatal if swallowed, inhaled, or absorbed through skin. Exposure to colchicine causes respiratory tract irritation, skin irritation, eye irritation, and serious eye damage, and can be carcinogenic.

***Precautions to be taken:***

- To avoid exposure of workers to colchicine, a separate room should be assigned for storing colchicine powder and for colchicine solution preparation and treatment.
- The laboratory should be equipped with a fume hood to handle colchicine and an exhaust fan to remove chemical odors and vapors.
- A cart should be specifically assigned to the lab to move solutions.
- Colchicine should be stored in a refrigerator, which should be securely locked.
- The process of colchicine treatment should be automated as much as possible to reduce exposure.
- Material safety data sheets should be easily accessible in the lab for all the chemicals used.
- Persons working with colchicine should wear protective gloves, respiratory protection, eye protection, and whole body cover.
- Workers should wash hands thoroughly every time after handling colchicine.
- Colchicine waste should be stored in a secluded room, which should be locked.
- Waste should be disposed of by a well-trained hazardous waste disposal team.
  In case of exposure, the exposed part should be rinsed cautiously with water for several minutes. Immediately call a poison center or a doctor with experience in occupational safety. Material safety documents should be presented to the doctor.

# 6. Putative DH Seedlings: From the Lab to the Field

*George Mahuku*

Management of haploid seedlings is crucial for the success of doubled haploid (DH) line development. There are two critical steps: (1) managing colchicine-treated D0 seedlings and reestablishing these seedlings under greenhouse conditions; and (2) managing putative DH plants under field conditions. At each of these steps, loss of putative DH plants can occur, affecting the success rate of achieving DH lines. This chapter addresses some of the pertinent issues (handling of DH seedlings, availability of suitable facilities to raise DH plants for maintenance, and seed multiplication and optimizing handling of putative DH lines under greenhouse and field conditions) that are required for optimal recovery of DH lines.

**Managing D0 Seedlings**

After treating the haploid seedlings with colchicine, drain the solution from the treatment container and collect it in specially designated residual waste containers. Rinse the treated seedlings with tap water at least thrice to remove residual colchicine. Rinsing is performed by filling the treatment container with tap water until all seedlings are fully submerged, followed by draining and collection of the waste solution into special toxic waste containers for proper disposal. A final wash/rinse should be conducted using 100 ppm of chlorox, which acts as a disinfectant and minimizes contamination of seeds by bacteria and fungi. After this, the seedlings are ready for transplanting in the greenhouse.

***Note:*** *All the colchicine waste must be collected in specially designated and clearly labeled container(s) and disposed of by an authorized company/agency. Please follow the relevant rules and regulations for safe disposal of dangerous chemical wastes, as applicable in your specific institution and country. Under no circumstances should these toxic residuals be disposed of through the common sink!*

**Handling treated seedlings**: Take utmost care while handling the seedlings, especially after treatment. The coleoptile is a very vulnerable tissue and could easily break if not handled properly. Therefore, handle the seedlings by holding the kernel, and do not touch the root or coleoptile to avoid possible breakage. Damage to the tissue during preparation of seedlings for treatment or during the subsequent handling of treated seedlings can lead to necrotic shoot tissue and subsequent seedling death.

***Transplanting materials*:**

- **Colchicine treated seedlings:** Each population should be clearly labeled to avoid misidentification while transplanting.
- **Tray with sterile distilled water:** Seedlings should be transported to the greenhouse in a tray with water to avoid dehydration.
- **Lab coats, gloves, and work suits:** Remember that seedlings were treated with colchicine; so take adequate operational health and safety measures while handling the treated seedlings.
- **Greenhouse or screen house**: This should have controlled conditions (temperature, light, and humidity).
- **Labeling stacks**: These are required for proper identification of the populations being transplanted.
- **Masking tape and permanent markers:** For recording all necessary information.

- **Jiffy pots (in trays) with potting medium**: Use greenhouse soil (peat moss) if possible, but any soil can be used as long as it is properly sterilized. Use soil with high organic matter content and avoid soil with a high clay content, if possible. In CIMMYT, we use either promix or premier peat moss. The promix is more compressed and is ready to use, while the peat moss should be mixed with at least 10% perlite (agrolita).
- **Greenhouse soil:** Use sterile soil with high organic matter.

***Transplanting procedure*:**

- Take the treated and washed seedlings to the greenhouse for transplanting, making sure that they are in a tray with water to avoid dehydration.
- Different types of pots can be used (see below) for transplanting; the choice depends on the budget, availability of materials, and transplanting methods.
- First fill each pot about halfway with soil (see below for the type of soil). Then carefully place the seedling (holding the attached seed rather than the shoot or root) onto the soil and hold it while filling more soil around it until the pot is well filled and only the tip of the coleoptile is visible (Figure 1). Gently push to make soil compact and prevent soil run-off during watering. Leaving a large part of the elongated coleoptile outside will increase the chance of damage and will reduce the number of plants that can successfully be transplanted in the field.
- Care should be taken to avoid or minimize breaking the coleoptile, as seedlings are very fragile and break easily after treatment. While transplanting, make sure that the size of the coleoptile outside the soil is less than 2 cm, as any length greater than this may increase the risk of coleoptile breakage and thereby affect the number of successfully established DH plants.

***Note:*** *Protective gloves should be worn all the time when handling colchicine-treated seedlings!*


**Figure 1.** Transplanting treated putative DH (D0) seedlings into jiffy pots in the greenhouse. Make sure that only the tip of the coleoptile is visible so as to avoid damage and loss of plants. [Photos: G. Mahuku]

**Managing the D0 greenhouse**

**Types of pots for greenhouse transplanting**: Various types of pots can be used: (1) typical plastic pots (approximately 5×5×8 cm) commonly used for greenhouse experiments and horticulture that are made of durable plastic and can be reused; (2) Styrofoam cups, more commonly used for coffee or tea, which are very cheap but less durable; or (3) pots made of biodegradable material that decays in the soil allowing transplanting of seedlings along with pots and thereby enabling the use of a planting machine (Figure 2). All pots must have perforated bottoms to allow drainage of excess water.

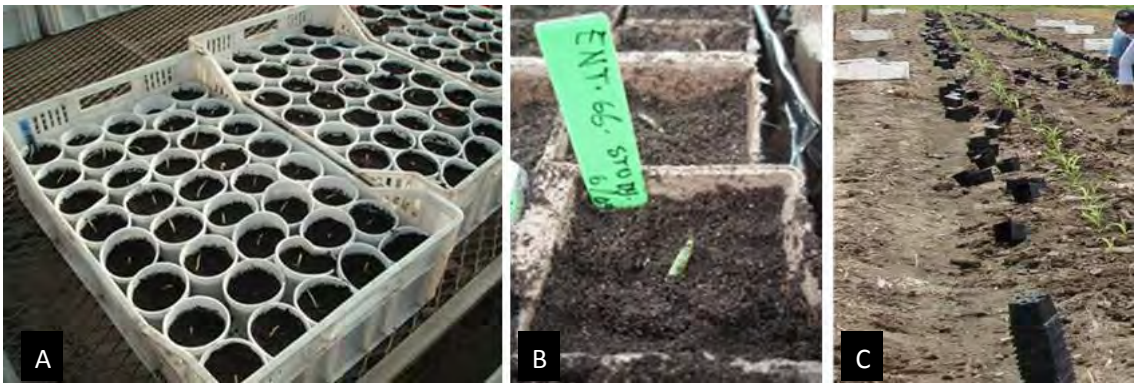


**Figure 2.** Transplanting treated seedlings into pots: (A) Styrofoam cups normally used for coffee, (B) jiffy pots, and (C) plastic pots. The pots are filled with sterile soil containing high organic matter content. Pots are placed in special containers for easy handling and management. [Photos A & B: V. Prigge, photo C: G. Mahuku]

**Maintenance of seedlings in the greenhouse**: Place the potted seedlings in special containers in the greenhouse (Figure 2) for approximately 10 days to two weeks so that they recover from the colchicine treatment and grow to the three- or four-leaf stage. It is important that proper fertilization and control of insects and diseases is undertaken so that the treated plants recover well and are vigorous. During this period, maintain the following conditions favorable for seedling growth:

- Keep the soil moist but avoid excess water. Watering can be done once per day or as needed.
- Apply the required dose of fertilizer in a soluble form with the irrigation water. It is also advisable to use a fertilizer with high phosphorus content to stimulate root growth.
- Three days after transplanting, fertilize plants with Triple 20 – this may be done by dissolving 2 tablespoons of fertilizer in 20 liters of water and using this to irrigate plants until they are ready for field transplanting.
- A week after transplanting, do a foliar application of Hakaphos violet (13-40-13; NPK), at a rate of 2 grams per 1 liter of water. Hakaphos stimulates root development and growth.
- Apply Gaucho (a systemic insecticide) 10 days after transplanting or a week before transplanting in the field; use just enough water to wet the trays without having an overflow. Gaucho is applied at 2.4 grams per 20 liters of water; the quantity depends on the number of plants to be treated.

**Greenhouse conditions:** Greenhouse conditions should be optimal for plant growth. Temperature should be maintained at less than 30°C and should not go below 20°C at night. Too high or too low temperatures will stress the plants and affect plant establishment and development. Use data loggers, if possible, to monitor temperatures and relative humidity within the greenhouse.

**Transplanting D0 seedlings to the field**

**Field conditions:** Selection of a proper site is crucial to the success of DH line development. If possible, select a site that has no or minimal disease and insect pressure. Temperatures should seldom go above 30°C and night temperatures should not go below 20°C during the growing cycle of the plants. Therefore, it is important to have data loggers in the field, to monitor the climatic variables (Figure 3). If light intensity is too high, use 50% aluminet shade cloth (http://www.greenhousemegastore.com/Stock-Shadecloth-50-Aluminet/productinfo/SC-ST50A/); this will both shade the plants and reduce the temperature underneath the shade cloth.

***Note***: There is aluminet for external use and for internal use inside greenhouses. Aluminum cloth shading will reduce light intensity by 50%, and this will help with plant establishment and pollen set. The cloth should be put 4 to 5 meters above the plants and leave enough space so that the tract operations can be done inside the field. Also, make sure that there is very good air circulation; otherwise temperatures can rise and this will affect pollen set and shedding.


**Figure 3.** Agronomic management of D0 nursery to minimize stress on the plants: (A) 50% aluminet shade cloth used to reduce the light intensity and temperature so as to avoid stressing the plants and avoid tassel blasting. The shading cloth is put 4 meters high to allow proper circulation of air, movement of tractors, etc. (B) Data logger for measuring light intensity and temperature. (C) Hobo data logger for measuring relative humidity and temperature. The data loggers are programmed to record every 30 minutes. [Photos: G. Mahuku]

***Materials:***

- **Seedlings**: These should be two-week seedlings previously established in the greenhouse.
- **Plastic storage containers:** These are necessary for transporting seedlings to the field while minimizing damage to plants.
- **Prepared field ready for establishing the D0 nursery:** The land should preferably have drip irrigation and plastic sheeting established. If necessary, shading cloth should also be put in place.
- **Transport to carry seedlings to the field:** Depending on the number of seedlings to be handled, one can use a tractor-mounted trailer or a pickup truck.

**Transplanting to the field**: Well-established seedlings should be transplanted in the field after two weeks (maximum), as follows:

- Take out the seedlings from the greenhouse and put them in a screen house close to the D0 nursery; leave the seedlings for one to three days so that they acclimatize to field conditions.
- Transport the seedlings in plastic trays using a tractor or pickup truck, to minimize damage or stress to the plants.
- Organize the seedlings according to population and transplant them together, so as to avoid confusion and mixing of populations.
- Water the seedlings well, approximately one hour before transplanting.
- Transplanting should be conducted early in the morning to avoid mid-day high temperatures, and the transplanted plants should be irrigated immediately to avoid stressing them. If soils have a high clay content, jiffy pots may not degrade well and thus will create a stressed environment for the plants. In such instances, eliminate the jiffy pots just before planting.
- Immediately after transplanting (or when a row has been completed), open the drip irrigation valve and start watering.
- Make a list of the total number of plants that have been transplanted.

***Note:*** *Field transplanting can be done manually or with a transplanter* (Figure 4). *This is convenient especially for large-scale applications.*


**Figure 4.** Depending on soil type, availability of labor, and size of the populations, transplanting seedlings in the field can be done either manually (A) or mechanically (B) using a tractor mounted planter. [Photos: G. Mahuku]

**Agronomic management**: This is the single most critical factor for successful recovery of D0 seedlings along the DH line development pipeline. If this process is not managed well, success rates will be low, even if the other steps were successfully executed. Optimization of irrigation regime, fertilizer application, and effective management of weeds, diseases, and insects are crucial for minimizing stress on the D0 plant. As the D0 plants are weak from the start, any type of stress will contribute to reducing the success and recovery rate of DH lines. Optimum climatic conditions are required; where possible, select a site that meets those conditions, soil type, and fertility regimes with minimal or no pressure from insects and diseases. Timely application of inputs such as irrigation, fertilizer, herbicides, and pesticides is critical for proper plant establishment. Shading with nets can be very helpful during anthesis, particularly when temperatures are abnormally high. Shading nets will reduce temperature and radiation stress to plants (Figure 3).

***Irrigation****:* D0 seedlings have weak roots and use much less water than a normal inbred or hybrid. Therefore, it is crucial that the right amount of water is applied for optimal plant development. Too little water will stress the plants and affect normal establishment and development. Too much water will result in chlorotic lines with thin stalks, and this will affect subsequent cob size and pollen production. Depending on soil type and water holding capacity, a proper irrigation schedule should be worked out that optimizes water and nutrient use efficiency. In this regard, drip irrigation is particularly suitable in the D0 nursery; where possible, this should be accompanied by probes at various points in the D0 nursery, to monitor soil moisture and assist in proper scheduling of irrigation regimes (Figure 5).

**Figure 5.** Drip irrigation to manage water application in the D0 nursery. Fertilizers can also be effectively applied using the drip irrigation system. [Photos: G. Mahuku]

***Proper fertilization:*** This is critical to plant development, and where the drip irrigation is being used, this should be applied as a solution along with the irrigation water. The first irrigation following transplanting should contain high phosphorus fertilizer [e.g., Haifa polyfeed drip 13-36-13 or Peter's Professional 9-45-15 (NPK)] for improved root development and plant establishment. Too much water or heavy rains can affect nutrient availability, as most will be leached out. This can be problematic during rainy seasons and if there are frequent rains, fertilizer should be banded, and avoid saturating the soil. Plastic sheeting and growing the plants on raised beds will minimize this problem. Where drip irrigation is being used, connect a fertilizer tank to the main irrigation by a venturi valve, calculate the quantity of fertilizer you want to apply per hectare, and feed a concentrated solution in the irrigation water. Micronutrients are crucial to improved plant establishment and subsequent flowering promotion. These should be foliar-applied during the entire plant growth period, following the recommended dosages and frequency of applications. During land preparation, 75% N, 100% P, 100% K is incorporated into soil, and this is also applied through drip irrigation just before flowering. Foliar application of nutrients is essential to enhance plant growth and development. Three days after transplanting**,** Hakaphos Violet A 13–40–13 (NPK) is applied at 2.4 grams for 20 liters of water once every week, and Impulsor at 40 ml per 15 liters of water (at the rate of 0.75 liters Impulsor per hectare). It is important to consistently monitor the plants and apply foliar nutrition or fertilizers as needed.

***Weed control***: Proper weed control is essential, to avoid competition and maximize nutrient availability and use by D0 plants. Hand weeding is preferred and where possible minimizes the use of herbicides, as most D0 plants are highly sensitive to residual herbicides, and hence this will affect proper plant development and establishment. Plastic sheets (foils) are an excellent, low-cost way to manage weeds, and these are routinely used in horticultural crops. Apart from managing weeds, plastic foil will help regulate soil temperature and humidity within the rooting system, resulting in uniform DH plant establishment and growth. Drip irrigation tubing and plastic sheeting can be placed in one step, using a tractor-mounted device (Figure 6).

**Figure 6.** Plastic foil is used to better manage weeds and regulate soil moisture and humidity. DH plants under plastic sheeting were found to perform better than those without plastic covers. [Photos: G. Mahuku]

**Disease and insect control:** In the tropics, disease and insect pressure is a major problem that can affect the rate of recovery of DH lines. A judicious insect and disease management regime is required to minimize plant damage and increase the rate of DH line recovery. The time of fungicide and insecticide application is crucial to minimize the effect on flowering, especially pollen shedding. Stop fungicide and insecticide two weeks before flowering to minimize the possible effects on flowering. Depending on the incidence of diseases and insect pests, use the recommended fungicides or pesticides. Foliar diseases

such as Northern and Southern Corn Leaf Blights are controlled using the fungicide Tilt (PROPICONAZOLE), which is applied one month after planting or when symptoms are noticed, and thereafter once every two weeks at 0.75 liters per hectare. This fungicide is effective against most foliar diseases, but application should be stopped a week or two before flowering. Gaucho is a systemic insecticide that is applied during the seedling stage and will protect plants from most insects (Figure 7). Cutworms are controlled using Lorsban Grana lade 3% insecticide (40 kg/ha), and this is incorporated into the rows during land preparation. Stem borers are controlled using Lorsban 480 EM at a rate of 1 liter per hectare or using Karate Zeon at 0.5 liters per hectare.

***Note***: Mention of specific brand names of commercial chemicals (including fertilizers, fungicides, and pesticides) is not intended as an official endorsement of the product by CIMMYT. There may be other equal or better products available in the market for achieving the same task.


**Figure 7.** Ear worms can be devastating in the D0 nursery, and a judiciary schedule for managing such insect pests is required. The figure shows the damage that can be done by (A) corn ear worm (*Heliothis* sp.) and (B) insecticide application to manage the pest. [Photos: G. Mahuku]

**Self-pollination of D0 plants for deriving new DH lines** In this step, the putative doubled haploid (D0) plants are carefully self-pollinated to derive the D1 seed, which in essence is the new DH line for further seed multiplication or use by the breeder. Please note that colchicine treatment may or may not lead to uniform or complete doubling of the chromosomes of all cells of a seedling; this is called "sectoral diploidization." The effect may be variable, especially on the genotype and the colchicine application. Some plants may have tassels producing abundant pollen while, in most instances, tassels may have limited pollen-producing anthers or none at all (Figure 8). Consequently, self-pollination may prove difficult. Therefore, well-trained staff are required to avoid losing such genotypes due to unsuccessful self-pollination.


**Figure 8.** Pollen production by a putative DH plant: (A) good quantity of pollen produced; (B) only a few branches are producing pollen while the rest are sterile due to sectoral diploidization; and (C) a sterile tassel, a common problem that could be observed in the D0 nursery. [Photos: G. Mahuku]

**Identifying and discarding "false" (diploid) plants in the D0 nursery:** Misclassification of haploid kernels can sometimes result from insufficient expression of phenotypic color markers, presence of dominant anthocyanin color inhibitor genes, and lack of trained personnel. However, putative DH plants can easily be distinguished from normal diploid plants under field conditions. The DH plants can be differentiated from the normal diploid plants on the basis of plant vigor, leaf habit, tassel size, and anthocyanin pigmentation on the stalk. Monitor coloration of the stalk of putative DH plants before flowering time and eliminate plants that show purple stalk coloration. False plants need to be discarded in time to avoid competition for light, water, and nutrients, avoiding pollen contamination to correct DH plants, and focusing the efforts on correct (DH) plants for maintenance.

**Self-pollination for maintenance and seed multiplication of new DH lines**

**Materials:**
1. Custom-made glassine bags (or "silk bags") and common pollination bags (or "tassel bags," Lawson No. 501).
2. Trained and dedicated personnel

**Monitor anther emergence**: Well-trained field staff are crucial to perform this step. The DH plants are generally weak, often have only a few pollen-shedding anthers, and may only shed limited pollen for a few days. Hence, constant monitoring to spot the D0 plants shedding pollen (among the many plants which may not have fertile tassels), immediately collecting the pollen and performing the self-pollination are critical for recovery of D1 seed and DH line development. It should be noted that the DH genotype will be lost if self-pollination is not properly undertaken, even if all the previous steps are performed perfectly. Pollination is a labor-intensive step, and during this period, a skilled workforce must be constantly in the field to avoid missing any plant that is ready for pollination.

***Note:*** *Success of DH operations depends on well-trained staff, especially the lab and field workers. Experience does matter, and with each cycle efficiency is increased. Therefore, avoid high staff turnover as this can significantly affect the success rates.*

**Pollination:** Cover the ear shoots before any silk emergence. Non-coated, transparent glassine bags or "silk bags" (approximately 6×20 cm) are most suitable to collect pollen from the putative DH plants for self-pollination. As pollen production is often limited in these plants, the transparent bags allow visual assessment of the quantity of pollen collected for self-pollination. If necessary, the pollination can be repeated the next day. After successful pollination, common pollination bags or "tassel bags" can be used to cover and protect the pollinated ears. For pollination, cover tassels with pollination bags in time before the intended pollination, and try to self-pollinate each plant. Cover the pollinated ears properly with tassel bags for protection and fasten them tightly with stapler pins (Figure 9).


**Figure 9.** Pollination in the D0 nursery: (A) non-coated, transparent glassine bags or "silk bags" are used to collect pollen from putative DH plants for self-pollination; (B) a successfully pollinated plant. [Photos: G. Mahuku]

**Harvesting self-pollinated ears after physiological maturity**: Often only few seeds are set on the ear of a DH plant (Figure 10). Therefore, utmost care is needed to avoid seed loss in the field. The ears should be harvested carefully and kept in proper cover bags during transport to the warehouse for dehusking and drying. This seed represents the newly developed, completely homozygous DH line, which may be planted again for seed multiplication to be able to use the DH line in further research and breeding activities. If there are some ears bearing purple-colored seeds, discard these because they are misclassified plants (normal diploids and not DH) that could have been missed in the earlier steps. Seed production on DH plants is expected to improve in subsequent cycles of DH production because (1) selection occurs in source germplasm for genes imparting favorable response to haploid induction and artificial chromosome doubling, and (2) the technical and field personnel involved in DH operations gain experience.


**Figure 10.** Harvested ears from the D0 nursery. There could be considerable variation in the amount of seed produced on the D1 (DH) ears, varying from one or two seeds to more than 50. Therefore, adequate care should be taken to avoid any loss of seed while harvesting. [Photos: G. Mahuku]

# 7. Integrating Marker-Assisted Selection in the DH-Based Breeding Pipeline for Rapid Development and Delivery of Superior Parental Lines and Cultivars

*R Babu, Sudha K Nair, BS Vivek, Felix San Vicente, and BM Prasanna*

**Introduction**

In recent years, doubled haploids (DH) and molecular markers have emerged as two of the most powerful technologies that are revolutionizing the way homozygous lines are developed in applied maize breeding programs (Mayor and Bernardo, 2009). As discussed earlier in this manual, the DH technology significantly reduces the time required to obtain genetically homozygous and pure lines compared to conventional inbreeding. Important advantages of using DH lines in the breeding program include a maximum genetic variance between selection units and an increased precision in estimating the genotypic value of DH lines and their testcrosses (TCs) (Gordillo and Geiger, 2008). In addition, utilizing DH lines in the breeding program permits early selection of prospective hybrids, simplifies the logistics of inbred seed increase and maintenance, and allows quick fixation of favorable alleles at quantitative trait loci (QTL) (Mayor and Bernardo, 2009; Lubberstedt and Ursula , 2012). When coupled with seed DNA-based genotyping (Gao et al., 2009), especially for large effect genomic regions conditioning nutritional quality (e.g., *crtRB1*-governed beta carotene content) or disease resistance traits (e.g., *msv1*-driven Maize Streak Virus resistance), DH-based molecular breeding results in enormous saving of time, labor, land, and other resources.

Line development and recurrent selection (RS)-based population improvement are the two most routinely applied activities in maize breeding programs. The improved source populations obtained through RS are used either as new source germplasm for deriving inbred lines or directly as synthetics that could be released for farmer cultivation in resource-poor regions. Here, we discuss two possible and pragmatic approaches to integrating marker-assisted selection (MAS) strategies in DH-based breeding programs.

**Integrating MAS in DH-based pedigree breeding pipeline**

Pedigree breeding along with extensive multi-location testing across a wide range of target environments has been the mainstay of maize improvement programs worldwide. Pedigree breeding starts with crossing of two elite genotypes that have complementary traits (such as good agronomy, abiotic stress tolerance, disease resistance, and nutritional quality), and in the successive generations, superior progenies combining the different desirable traits are selected until homozygosity is achieved in F7 or F8 generation. A selection history is maintained throughout the breeding generations. With the advent of DH technology, it is possible to obtain homozygous lines in only two generations as against the seven to eight generations that are mandatory in a typical conventional pedigree breeding. While the DH technology makes it possible to save time significantly, it removes, to a certain extent, the selection opportunities that a breeder generally has during multiple filial generations. In general, it has been a routine practice in maize breeding to induce haploids at F1 generation to save time. However, F1-derived doubled haploids tend to have reduced recombinations (because there is only one round of meiosis) and have been found to decrease the responses to single or multiple cycles of selection pressure (Riggs and Snape, 1977; Jannink and Abadie, 1999).

For a trait controlled by 100 or more QTLs, Bernardo (2009) reported, based on simulation experiments, that the cumulative responses to selection were up to 4–6% larger among F2-derived DH lines than among F1-derived DH lines and hence suggested inducing haploids from F2 rather than F1 for sustaining long-term enhanced selection response. However, deciding between F1 and F2 involves a certain trade-off between time and resources for the breeding program. As proposed by Bernardo (2009), if initial F1s

are made on a speculative basis in the breeding program, inducing haploids at F2 may not consume additional time.

Besides enhanced recombination, an important advantage of inducing haploids at F2 is the opportunity to subject F2 individuals (both at the seed and plant level) to required genotypic as well as phenotypic selection pressure. Depending on the target traits of the breeding program and availability of molecular marker information for the specific locus/loci governing those traits, F2 seeds could be individually seed-genotyped through non-destructive sampling (Gao et al., 2009), and the seeds carrying unfavorable alleles in homozygous condition could be discarded. This procedure has been described as "F2-enrichment" (Howes et al., 1998; Bonnett et al., 2005; Wang et al., 2007). Currently in maize, few loci have been identified that confer large phenotypic effects for which such an approach would be feasible. Some examples are as follows:

- *Crtrb1*, a carotene hydroxylase gene (Yan et al., 2010) has been demonstrated to have a 2- to 10-fold effect on beta-carotene content across diverse genetic backgrounds in the tropical maize germplasm (Babu et al., forthcoming). Seed DNA based genotyping of *crtRB1*, especially in the upstream generations such as F2 or F3, is routinely employed in the HarvestPlus–Maize breeding program at CIMMYT, which has paid rich dividends leading to development of lines that have significantly higher levels (15–20 ppm) of provitamin A as compared to 1–2 ppm found in normal yellow maize.
- *Opaque2* (*o2*) is a transcriptional regulator in maize whose mutant allele confers twice as much lysine and tryptophan in the endosperm as normal maize, which along with associated endosperm modifiers is known as Quality Protein Maize (QPM) (Prasanna et al., 2001). Molecular markers located within *o2* have been successfully used in the rapid development of QPM versions of normal maize lines (Babu et al., 2005).
- Maize Streak Virus (MSV) is a major disease in most parts of sub-Saharan Africa; a large effect QTL conditioning MSV resistance has been identified on chr.1 (Welz et al., 1998; Lu et al., 1999; Kyetere et al., 1999) across different genetic backgrounds. The CIMMYT Global Maize Program has recently identified (and is presently validating) a set of SNP markers in this genomic region which could be potentially utilized for effective differentiation of MSV resistant and susceptible genotypes without phenotypic selection. Though additional minor loci influencing MSV resistance may exist in other parts of the maize genome or in different genetic backgrounds, *msv1* may be considered as an essential prerequisite for achieving reasonable levels of resistance to the disease (Sudha, personal communication).

With wider adoption of Genome-Wide Association Studies, the maize genetics community is likely to unravel and validate further a large number of marker-trait associations in the coming years, which is expected to enable F2 enrichment as a preferred approach in maize, thereby providing scope for pre-selecting source germplasm before DH induction. When the marker-selected individuals are grown in the field, additional phenotypic selection for general plant vigor, type, and other *per se* traits could be exercised, ensuring that only "good" genotypes are subjected to the DH induction procedure.

An illustrative DH-based MAS scheme is presented in Figure 1, which is aimed at combining drought tolerance with one of the disease resistance traits during pedigree breeding. As mentioned earlier, a large effect genomic region influencing MSV resistance has been identified and its phenotypic effect has been validated in diverse genetic backgrounds. Marker-based screening of individual F2 seeds for *msv1* could help in discarding individuals with an unfavorable *msv1* allele in homozygous condition, and further phenotypic screening for *per se* and plant vigor related traits will ensure elimination of weak plants from being subjected to haploid induction. Subsequently, the marker-screened and phenotypically selected plants are crossed to the tropicalized haploid inducer and putative haploid

kernels are identified. Upon chromosome doubling, and selfing of the D0 plants to D1 seed stage, simultaneously, pollen from the D0 plants (if available in adequate quantity) can be used for making TCs. Once sufficient DH-TCs are produced (using D0 or D1s), they can be evaluated for performance under drought and optimal conditions at multiple locations, representing target population of environments and best-bet drought tolerant hybrids combining reasonable levels of MSV resistance identified and nominated for National Performance Trials, and their corresponding parental lines maintained. Additionally, genotyping the DH lines enables estimation of marker effects for drought and optimal performances, which over the years can potentially contribute to genome-enabled prediction of untested DH lines, being generated year after year in the breeding program, thereby minimizing the managed screening/phenotyping requirements.

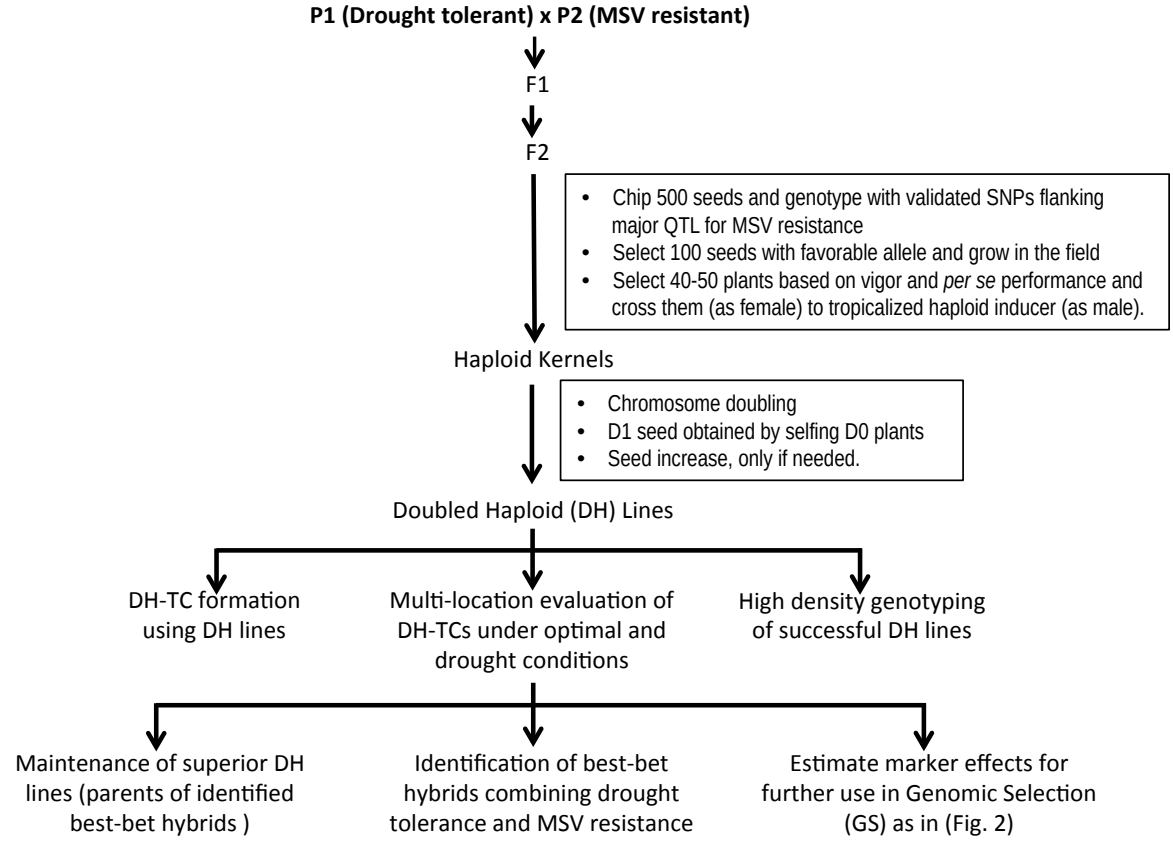


**Figure 1.** An illustrative scheme for DH-based, marker-assisted selection for potentially combining drought tolerance and disease resistance in a pedigree breeding program. MSV = Maize Streak Virus; D0 = putative DH seedling after chromosome doubling treatment of haploids; D1 = seed derived from D0 plants; TC = testcross.

**Rapid-cycle, open-source genomic selection**

Recurrent selection (RS) has been an important tool in maize breeding for developing improved source populations that are significantly enhanced in desirable allele frequencies, especially for highly complex, polygenic target traits like drought and heat tolerance. Despite being effective, RS based only on phenotypes is time consuming and resource demanding as it involves developing TC progenies and evaluation in replicated multiple locations before every selection step. Thus, if four RS cycles are intended, it entails a minimum of four seasons of TC generation and another four seasons of performance evaluation in multiple locations. Genomic selection (GS) is a novel approach that exploits high-density genotyping to predict the total genetic value of an individual based on a model set of training individuals that are phenotyped at representative locations. GS-based approaches typically

ignore information on the number and location of QTL and focus on the genetic improvement of quantitative traits rather than on understanding their genetic basis (Jannink et al., 2010). The usefulness of high-density genotyping based procedures that focus on predicting performance indicates that markers can be used as a selectable tool to improve a complex trait, even without a clear understanding of the underlying genetics of the trait. Rapid-cycle GS (RCGS) is a convenient tool to augment the pace of RS cycles without having to phenotype each set of intermated progenies. RCGS also saves a considerable amount of material resources as it involves only one season of phenotyping at representative locations. In the subsequent generations, intermated individuals are only genotyped and their genetic worth is predicted based on previously estimated marker effects.

An illustrative narration of RCGS in a closed multi-parent population context is presented in Figure 2. RS in a multi-parent population can be very effective as compared to in a bi-parental population, which is resource demanding and doesn't permit evaluation of a large number of populations. Typically, 8 to 12 elite maize lines with trait complementarity such as drought tolerance, disease resistance, and enhanced nutritional quality are chosen within each heterotic group and half-diallels are made so as to obtain all possible combinations. The F1s are intermated either in isolation or through controlled pollination to form a large F2 population. Depending on the target traits for the particular agro-ecology and availability of molecular information for such traits, F2 enrichment could be pursued as described earlier in the pedigree breeding context. In this illustration, F2 seeds are screened with four validated SNPs, which are flanking a disease resistance QTL and a major gene, *crtRB1*, which enhances beta-carotene content in the maize endosperm.

In the subsequent season, following F2 screening, at least 500 S2 families (C0) will be established for each multi-parent population, which will be genotyped and testcrossed. In the following seasons, TCs will be phenotyped under drought and optimal conditions in representative locations, and marker/haplotype/QTL effects will be estimated. The top 5–10% of the C0 families will be selected based on the test cross data and recombined to form C1 (cycle 1). The individuals of C1 will be genotyped and based on the previously calculated C0 marker effects, GEBVs (Genomic Estimated Breeding Values) will be estimated and the top 5–10% of the GEBV-selected C1 individuals will be recombined to form C2, without phenotypic evaluation. This would be repeated for one more cycle until C3, wherein the GEBV-selected individuals will be crossed to a haploid inducer for generating DH lines. If the breeding program manages to obtain a large number of DH lines from C3, one way of selecting a smaller portion of superior lines without phenotypic evaluation could be based on GEBVs (marker effects of C0 + genotypic information of DH lines). The GEBV-selected DH lines may be distributed to different small and medium enterprises (SMEs) and national agricultural research system (NARS) partners for respective evaluation (*per se* and TCs) in their target production environments. The phenotypic information generated by the SME seed companies and NARS partners could be shared, which will contribute to revised or updated marker effects to aid in future predictions. The pre-selection of F2 individuals (using specific marker information for nutritional quality and/or disease resistance traits) coupled with multiple cycles of recurrent selection based on robust marker effect estimates for drought and optimal conditions ensure that the C3-derived DH lines are superior in nutritional quality and disease resistance as well as competent in terms of performance under drought without yield penalty in optimum conditions. The open-source nature of the proposed scheme also ensures that the phenotype information generated by different partner institutions is shared while the institutions maintain proprietary rights over the material resources. The improved source population, C3, can also be shared with the interested NARS, which in turn may promote this as superior synthetic/OPV for farmer cultivation or use it in their own breeding program for deriving superior inbred lines. One can derive greater benefit from the proposed scheme when year-round nurseries are used for accelerating the breeding cycle.

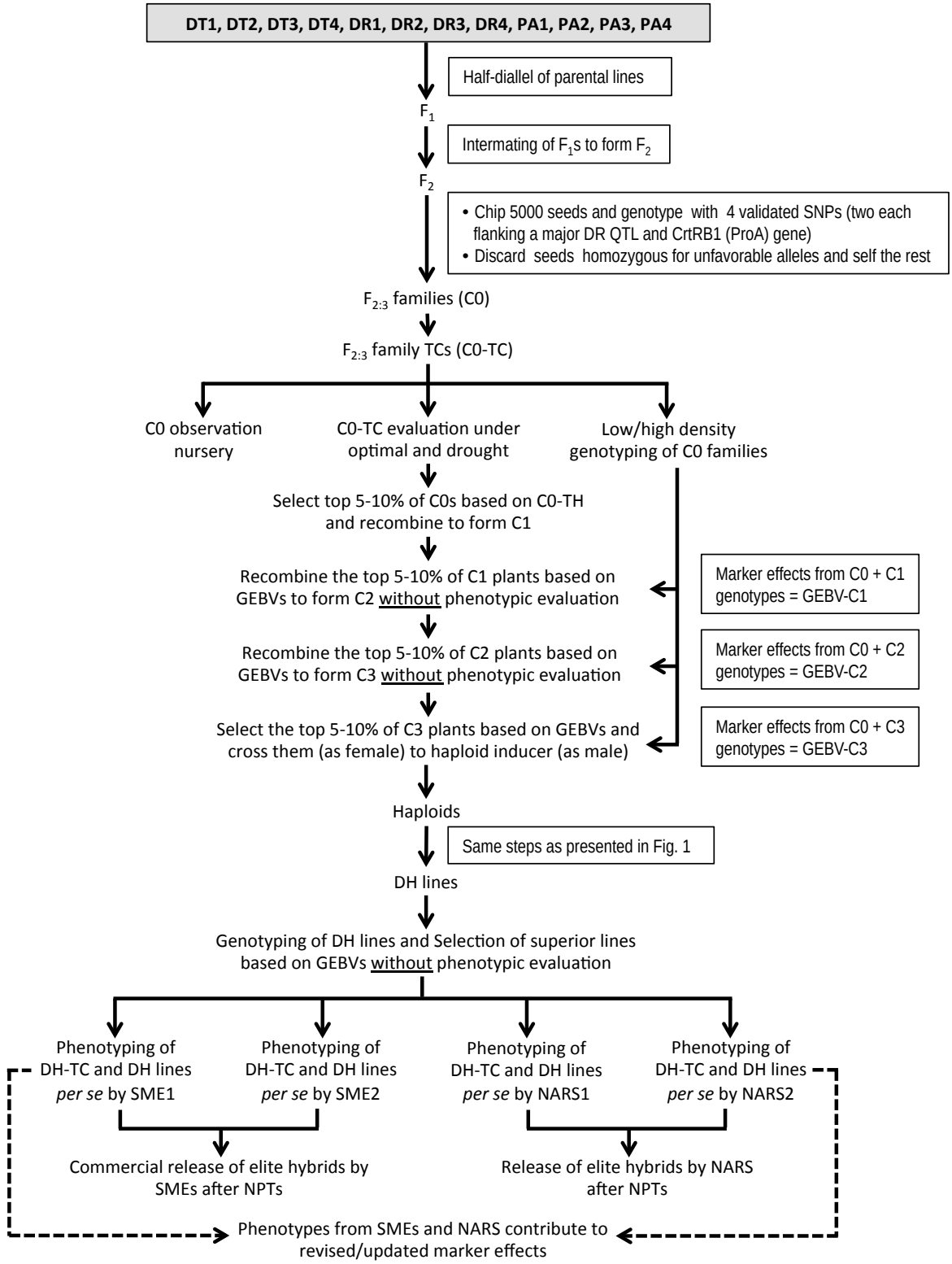


**Figure 2.** An open-source, multi-parent RS model for integrating molecular markers and DH technology to rapidly deliver superior lines with drought tolerance, disease resistance, and nutritional quality. DT = drought tolerance; DR = disease resistance; PA = provitamin A; C0 = cycle 0; C1 = cycle 1; C2 = cycle 2; C3 = cycle 3; TC = testcross; GEBV = genomic estimated breeding value; SME = small and medium enterprises; NARS = national agricultural research system; NPTs = national performance trials.

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# 8. DH in Commercial Maize Breeding: Phenotypic Selections

*Daniel Jeffers and George Mahuku*

**Introduction**

The use of doubled haploids (DH) in commercial maize breeding programs offered several benefits to the seed industry, including reduction of costs related to running the breeding operations, accelerated breeding cycles to bring products to market, and improved efficiencies in characterization and exploitation of new germplasm. DH technology has thus become a key component of the product development process of the large seed companies. This chapter will discuss how the use of DH, coupled with high-throughput and reasonably precise phenotyping, is being used in the commercial breeding programs.

Doubled haploids in maize have been produced for maize breeding since 1940s in the US (Chase, 1947, 1949), and as parental lines of commercial hybrids since the early 1960s (Troyer, 2004; Forster and Thomas, 2005). DeKalb 640 was the first widely-accepted, high density planting tolerant commercial hybrid in the US, and contained three DH lines in its pedigree (Chang and Coe, 2009). Though the induction rate and chromosome doubling rate initially occurred at a low frequency, the homozygous lines developed from elite pedigree breeding programs proved very useful in commercially oriented maize breeding programs.

For commercial breeding activities, speeding-up of the breeding cycle through DH technology has great benefit due to significant reduction in resources needed for line development. Commercial breeding companies, through the use of DH technology, also eliminate Stage 1 testing activities on early generation inbred lines. In as little as 3-4 seasons, following the development of D1 lines, the value of the new lines for use in commercial hybrids can be evaluated in stress screening nurseries and multi-location trials, and passed to the commercial seed production units of the company for use in pre-commercial hybrid strip plot testing. The breeding operations of several large multinational seed companies are currently based on the use of DH lines for majority of breeding activities. During 2011, Pioneer has reportedly generated more DH lines than the total number of inbreds generated in the first 80 years of their breeding efforts. This is representative of the multinational seed industry as a whole. The emphasis has now shifted on marker-assisted selection (MAS) and high throughput phenotyping of the newly generated DH lines.

**DH improves the capacity to identify breeding materials with superior performance under diverse environmental conditions**

In the large commercial breeding programs, the DH lines are now quickly screened using molecular markers and selections done, before further characterization for agronomic performance across many environments, and against relevant abiotic and biotic stresses. These include managed stress environments (Campos et al., 2004) that provide information on yield performance under less than optimum conditions. Evaluation of the completely homozygous DH lines and their hybrid products provide an excellent opportunity to link phenotypic performance with the genotype. Utilizing a commercial-scale DH program coupled with good phenotypic characterization for reaction to biotic diseases, Dow AgroSciences in Brazil rapidly shifted their elite inbred disease phenotypic profiles to multiple disease resistance by rapid recycling, and have developed a strong commercial pipeline of multiple disease resistant hybrids. This was done prior to the routine use of molecular tools to assist in genotyping the DH inbreds (D. Jeffers, personal information).

The large international seed companies in the last decade have made huge gains in their genotyping capacity, and realized that their ability to phenotype was not keeping pace (Campos et al., 2004). Therefore, heavy investments were made on improving high-throughput phenotyping capacity to evaluate maize germplasm under both optimum and stress conditions using "phenotyping platforms". The term "phenomics" has been used for the whole field of improved phenotypic characterization through the use of modern technology, including techniques such as digital imaging, spectral analysis, and canopy temperature measurements, linked with bioinformatics (Finkle, 2009; González-Pérez et al., 2011; Montes et al., 2011; Patil and Kumar, 2011). These techniques have been used to examine agronomic performance under optimum and stressed conditions, both for abiotic and biotic stresses, and provide a more quantitative measure of the responses of the germplasm. The improved precision has also provided the opportunity to better understand the genetic basis of response to various stresses.

**Linking DH with other technologies to accelerate breeding gains**

Doubled haploids are just one component of a technological package that has allowed commercial breeding programs to improve their breeding efficiency, and increase the genetic gains per breeding cycle. Examples from the seed industry are Pioneer's use of Accelerated Yield Technology, AYT™ System which encompasses molecular breeding, bioinformatics, doubled haploids, plant genomics, precision phenotyping and decision support tools to develop and deliver better commercial products. Precision phenotyping provides the capacity to examine the phenotypic response at the macro level, but also at the molecular level once an understanding of the genetic basis of response is known. Phenotyping tools such as "Proteomics" (Liebler, 2002) and "Metalabolomics" (Daviss, 2005) can then be used to better characterize the germplasm.

**Future perspective**

High throughput field-based phenotyping with reasonable precision plays a key role in the modern maize breeding operations, with significant advances in understanding the maize plant's response to its environment, and finally agronomic performance. As more information is obtained on the genetic basis of this response, molecular phenotyping will become a larger component of the phenotyping process that predicts genotypic response for commercial maize products. These activities can be carried out in large institutions including multinational seed companies, since it requires a significant investment in infrastructure. Haploid techniques can be a valuable tool for the rapid production of homozygous transgenic plants, thus assisting in the establishment of transformation techniques. Combining haploidy with other technologies, such as MAS, induced mutagenesis, and transgenic technology, would accelerate crop improvement. Doubled haploids will provide the products to facilitate these activities, and a rapid mechanism to deploy improved genetics in commercial products.

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# 9. Access to Tropicalized Haploid Inducers and DH Service to CIMMYT partners

*BM Prasanna, Vijay Chaikam, George Mahuku, and Rodrigo Sara*

**Access to tropicalized haploid inducers**

Adoption of DH technology by several of the maize breeding institutions under the national agricultural research systems (NARS) as well as small and medium enterprise seed companies, especially in the developing countries, is limited by the lack of inducers adapted to tropical/sub-tropical conditions. CIMMYT's Global Maize Program, in collaboration with the Institute of Plant Breeding, Seed Science and Population Genetics of the University of Hohenheim (UHo), addressed this limitation and now has haploid inducers ready for sharing with interested institutions, under specific terms and conditions as outlined below.

The tropically adapted inducer lines developed by CIMMYT and UHo have high haploid induction capacity (~8–10%) and were found to exhibit better agronomic performance compared to the temperate inducers in the CIMMYT experimental stations in Mexico (Agua Fría and Tlaltizapan). A haploid inducer hybrid developed using these TAILs revealed heterosis for plant vigor and pollen production characteristics under tropical conditions, while maintaining similar haploid induction rate (~8-10%). CIMMYT and UHo have decided to share the seed and grant authorization for use of one of the tropicalized haploid inducer lines (one of the parent of a hybrid inducer) and the hybrid inducer to interested applicants after signing of the relevant Material Transfer Agreement (MTA) and with certain restrictions to protect the intellectual property rights of both institutions for the inducer lines.

**Guidelines to obtain tropical haploid inducers**

The general guidelines to obtain inducers for research use and commercial use are as follows.

***For research use by NARS:*** The NARS institutions interested in accessing the haploid inducers for specific purposes, for example, for development of DH lines for use in breeding programs, may send a letter of intent or expression of interest to CIMMYT. For eligible NARS institutions, the haploid inducers will be provided free-of-charge by CIMMYT and UHo, after signing of a Research Use MTA. The use of the inducers by NARS institutions for their own commercial purposes or for commercial purposes of others should be in accordance with a separate license agreement for commercial use (as given below).

***For commercial use:*** Applicants may access the inducers for commercial use pursuant to signing of a Material Transfer and License Agreement with CIMMYT and UHo. Each applicant shall pay to UHo a one-time licence fee (US$ 25,000) for provision of seed of two haploid inducers; these include one of the parents of a tropicalized haploid inducer hybrid and the haploid inducer hybrid itself. If the applicant wishes to access the other parent of the haploid inducer hybrid, an additional one-time licence fee of $10,000 will be payable to UHo.

Seed of the above-mentioned haploid inducers will be provided by CIMMYT to the applicant normally within three weeks after signing of the MTA (for research use) or Material Transfer and License Agreement (for commercial use) and receipt of the one-time License fee, as relevant.

**Maize DH service by CIMMYT to International Maize Improvement Consortium (IMIC) partners**

CIMMYT recently established a maize DH production facility at its experimental station in Agua Fría, State of Puebla, Mexico. Through this facility, a DH line production service will be offered to members of the International Maize Improvement Consortiums operating in Asia and Latin America (i.e. IMIC-Asia and IMIC-LA) on a cost-recovery basis. For information as to how to become a Consortium member, please contact CIMMYT Global Maize Program Director.

At the Agua fría Station, the nursery for haploid induction in the source materials is planted in late May, and the seed is harvested by September. Haploid seeds are identified using kernel color markers, and the seedlings are subjected to chromosomal doubling immediately. The haploid (D0) nursery will be raised during November–April. The D1 seed of the DH lines will be processed and sent back to partners by May/June, following the necessary germplasm export protocol.

**Possible models for breeding programs to adopt DH technology**

***Model 1 – full service*:** Under this scenario, the partner sends in the source germplasm (populations for developing DH lines) and CIMMYT conducts all the steps (including inductions, classification, chromosome doubling, and D1 seed derivation) that are needed for DH line development. At the end of this process, CIMMYT sends all the seed of the DH line (D1 seed) that has been produced back to the client. For this scenario to work and be effective, the partner should consult CIMMYT in advance and express interest in sending populations for DH line development.

***Model 2 – partial service***: There are two possible scenarios under this model: (1) the partner does the induction of haploid kernels and sends the kernels for selection, chromosome doubling, and generation of DH lines in CIMMYT's centralized facility; or (2) the partner does the haploid induction as well as selection of haploid kernels, and sends only the haploid kernels for chromosome doubling and subsequent generation of DH lines (D1 seed) at the CIMMYT facility.

**How to indent for the DH service**

- At present, the DH service facilities at CIMMYT's Agua Fría station can handle a total of 150 populations per year, for meeting both the internal and external demands for DH line production. Interested partners can submit a maximum of 5 to 10 populations for haploid inductions and DH line generation.
- An announcement will be made each year in January inviting requests for DH line production. Partners wishing to utilize this service need to sign an MTA, with CIMMYT by the end of February.
- After signing the MTA and paying the necessary charge, as applicable, for cost recovery (as per the details given below), partners should send 200 seeds for each population for haploid inductions by the end of April. Along with the seed, partners need to provide flowering time information (especially silking) and adaptation (tropical/subtropical/highland).
- If partners wish to send only sorted haploid seeds for partial service (chromosome doubling and DH line generation), the haploid kernels should be sent to CIMMYT (after signing the MTA and paying the appropriate service fee) by 01 October at the latest.
- CIMMYT will inform the partners about success in production of DH lines from the source populations received, after haploid induction and DH line generation. In case a source population contains the kernel color inhibitor gene that prevents reliable identification of haploid kernels, CIMMYT will inform the concerned partner, and that specific source population will not be further continued for DH line production. In such cases, only haploid induction cost (US$ 200 per population) will be charged to the partner.

- Partners from private sector institutions need to pay the DH service fee after signing the MTA and before initiation of DH production work. Partners from public sector institutions may utilize the collaborator's budget for DH services before the MTA is signed. If the request is approved, service charges will be deducted internally in CIMMYT from the collaborator's budget, as applicable. Public partners without collaboration budgets should arrange the funding for DH service before the MTA is signed and work is initiated by CIMMYT.
- A cancellation cost will be charged for cancellation of any indented DH service work. The cost charged will be proportional to the amount of work already undertaken before the receipt of a formal letter from the partner requesting cancellation of the indented DH service.

**Cost recovery for DH line production service**

The costs for complete or partial service will be as indicated below:

1) *Only haploid induction:* US$ 200 will be charged for each source population subjected for haploid induction.
2) *Haploid seed identification and chromosome doubling:* US$ 25 will be charged for each DH line supplied.
3) *Only chromosome doubling and DH (D1)) seed production:* US$ 22 will be charged for each DH line supplied.
4) *Complete DH service (including haploid induction, haploid identification, chromosome doubling, and DH line production):* US$ 30 will be charged for each DH line supplied.

**Note:** These costs, solely from the cost recovery viewpoint, may be reconsidered and possibly revised by CIMMYT each year depending on operational costs.

**For further details, please contact**:

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