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Methods and Application of Double Haploid Technology in Maize Breeding: A Review

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Abstract

Maize is used for human and animal consumption, as a biofuel, and for a range of industrial purposes around the globe. In modern agriculture, farmers choose between two types of maize varieties hybrids and open-pollinated varieties and their choice depends primarily on the prevalent environmental and economic situation as well as the availability of seed of the preferred variety type. Therefore to supply improved varieties reducing the time needed for inbred development is the key role. This can be most effectively achieved by application of the doubled haploid (DH) technology. The objective of this topic is to review the method and application of DH technology in maize breeding. Producing DH lines typically requires four steps: inducing haploids by crossing heterozygous plants with a haploid inducer; identifying haploid kernels through morphological markers; chromosome doubling of haploids by colchicine treatment; and selfing to obtain seeds of DH lines. The utility of DHs in homozygous lines development, recurrent selection, induction of mutation, genetic enrichment, and integrity with other technologies make them the best material for genetic improvement and genetic studies of maize.

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Introduction

Maize (*Zea mays* L.*)* is used for human and animal consumption, as a biofuel, and for a range of industrial purposes around the globe. The crop is a food staple and, alongside wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.*)*, it is the main source of calories for the majority of people living in poverty-stricken areas of Africa, Asia, and America (Dowswell *et al.*, 1996).

Worldwide production of maize in 2021 was ~1.2 billion tons with the largest producer, the United States of America, producing 31% while Africa produced 7.8%. The consumption per capita of maize is 19.02 kg/capita/year worldwide, with Africa consuming about 43.2 kg/capita/year (FAOSTAT, 2021) although the continent produces less than 8% of the world's total.

Two major tools for meeting the future demands for maize and maize-based products are resource-conserving cropping systems and improved crop varieties that yield more with equal or less inputs than are currently used. In modern agriculture, farmers choose between two types of maize varieties hybrids and open-pollinated varieties and their choice depends primarily on the prevalent environmental and economic situation as well as the availability of seed of the preferred variety type (Pixley and Banziger, 2004). Hybrid varieties are commonly developed by crossing two unrelated, homozygous inbred lines.

Traditionally, the maize plants' cross-breeding nature required recurrent self-pollinations for 6 to 10 generations, i.e., 3–5 years when two seasons per year can be accomplished, to obtain sufficiently homozygous inbred lines Hallauer *et al.,* (2010). Hence, the key to increased genetic gains and accelerated development of improved varieties is reducing the time needed for inbred development. This can be most effectively achieved by application of the doubled haploid (DH) technology. The DH technology shortens the breeding cycle significantly by rapid development of completely homozygous lines in 2-3 generations, instead of conventional inbred line development process which takes at least 6-8 generations to derive lines ~99% homozygosity (Foster and Thomas, 2005; Chang and Coe, 2009). Haploids are plants (sporophytes) that contain a gametic chromosome number (n). They can originate spontaneously in nature or as a result of various induction techniques (Murovec *et al.,* 2012). Spontaneous development of haploid plants has been known since 1922, when Blakeslee first described this phenomenon in *Datura stramonium* (Blakeslee *et al.,* 1922); this was subsequently followed by similar reports in tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*) and several other species (Forster *et al.,* 2007).

Haploids in maize can be appearing spontaneously or they can be the result of various induction techniques. However, spontaneous occurrence is a rare event and therefore of limited practical value. Thus, various induction techniques have been studied and improved (Mureseanu *et al.,* 2013). Haploids can be obtained either through *in vitro* (androgenesis) or *in vivo* methods. The DH technology in maize breeding, based on *invivo* haploid induction, is recognized worldwide as an important means for enhancing breeding efficiency. The technology has become the backbone of many commercial maize breeding programs in Europe (Schmidt, 2003), USA (Seitz, 2005), and recently in China (Chen *et al.,* 2009). Factors making DHs increasingly attractive include the development of better inducer lines, more efficient chromosome doubling methods, and protocols to efficiently introgress transgenes, especially stacked transgenes (Prasanna *et al.,* 2010).

Several maize breeding institutions in the public sector, as well as small and medium enterprise 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. Therefore, the focus of this paper is to review the method and application of double haploid technology in maize breeding.

Production of Double haploid Lines in Maize

The importance of haploids and DHs in breeding and genetics studied was quickly realized and research was started to identify different methods and factors for improvement and high frequency of haploid production. Several Haploid techniques were identified for production of haploid and doubled haploids in maize breeding. A doubled haploid (DH) genotype is formed when haploid *(n)* cells undergo either artificial or spontaneous chromosome doubling producing two gene sets which are exactly identical (Prasanna *et al.,* 2012). Barnabás *et al.,* (1999) and Maluszynski *et al.,* (2003) reported that DHs can be produced artificially through *in vivo* or *in vitro* techniques under different frequencies.

The *in vivo* approach is focused on parthenogenesis and pseudogamy (chromosome elimination after wide crossing). The haploid embryo is then rescued, cultured and chromosome doubled to produce doubled haploids. The *in vitro* procedure focuses on gynogenesis (ovary and flower culture) and androgenesis (anther and microspore culture). Genetic analyses generated by several workers have shown that *in vitro* androgenetic response is under complex multifactorial control. Thus, despite good results with specific genotypes, the technique has not yet become a routine tool in maize breeding. In contrast, *in vivo* procedures have been widely applied during the last two decades since they can be improved considerably. Producing DH lines typically requires four steps: (i) inducing haploids by crossing heterozygous plants with a haploid inducer; (ii) identifying haploid kernels through morphological markers; (iii) chromosome doubling of haploids by colchicine treatment; and (iv) selfing to obtain seeds of DH lines (Melchinger *et al.,* 2005; Seitz, 2005).

Induction of Haploids

In vivo Maternal Haploid Induction in maize

In vivo haploid induction has been highly successful in maize and is now extensively followed by several commercial breeding programs (Prasanna *et al.,* 2012). 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 Stock6 (Coe, 1959) and further derivation of an array of maternal haploid inducers in maize, revolutionized the application of DH technology in maize breeding, as this method is much less dependent on the donor genotypes from which DH lines are derived.

Two modes of *in vivo* haploid induction are recognized; maternal and paternal haploids (Rober *et al.,* 2005; Zhang *et al.,* 2008). For maize (*Zea mays* L.) the most used induction technique is *in vivo* induction of maternal haploids. This technique is based on the use of special genotypes that have the characteristic to induce haploids.

Two hypotheses have been proposed about the mechanisms of maternal haploid induction. The first hypothesis supported by Wedzony *et al.,* (2002) states that one of the two sperm cells coming from the inducer line pollen is defective but still capable of fusing to the egg cell. During cell division, the chromosomes from the inducer parent deteriorate and are eliminated from the primordial cells. The second hypothesis supported by Chalyk *et al.,* (2003) states that one of the two sperm cells is unable to fuse with the egg cell. As a result of this phenomenon, haploid embryogenesis is activated. The second sperm cell then fuses with the central cell. The resulted haploids are small, present low plant vigor and are sterile. In order to propagate them, their fertility must be restored. This can be obtained by spontaneous doubling of the chromosomes or by induced doubling. The resulted doubled haploids (DH) are completely homozygous and homogeneous (Chalyk *et al.,* 2003).

In vivo haploid induction ability in maize is a selectable trait and significant progress in the development of new inducers has been made during the last two decades. Public inducer genotypes for maternal haploids were developed at various international institutions and cover a range of HIR, such as: 2% for Stock6 (Coe, 1959), the first germplasm ever reported to induce haploidy and ancestor of all current inducers, and 8–10% for RWS (Rober *et al.,* 2005), HZI (Zhang *et al.,* 2008) and PHI (Rotarenco *et al.,* 2010). However, all of the above inducers were developed from temperate germplasm and principally evaluated for HIR and agronomic performance under temperate climatic conditions, CIMMYT global maize program has been developed tropically adapted inducer lines (TAILs; with 8–10% HIR) in collaboration with the University of Hohenheim, Germany (Prigge *et al.,* 2011).

In vitro Haploid Induction in maize

Haploids in maize can be obtained through *in vitro* (androgenesis). Androgenesis refers to the development of haploid plants from immature pollen either by Anther culture or microspore culture (Prasanna, 2012). In anther culture systems, microspores within the anther are induced to undergo androgenesis to form microspore-derived embryo-like structures. Embryo---like structures can either directly regenerate into haploid plants or indirectly regenerate via the formation of regenerablecalli. 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 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 (Genovesi and Collins, 1982; Spitko *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; Genovesi and Collins, 1982; Spitko *et al.,* 2006). Therefore, *in vitro* approaches for DH development are not very commonly used in maize.

Haploid Identification

Maize breeders commonly use color markers to differentiate kernels resulting from regular fertilization and those generated by haploid induction. Different methods have been identified to differentiate haploid from diploid kernels (Odiyo, 2013). Chase (1969) identified a phenotypic marker system based on anthocyanin coloration encoded by the dominant variant allele *R1-nj(R1-navajo)* of the *R1* locus. Integration of anthocyanin markers in haploid inducer lines facilitates haploid identification at the seed level and also during other stages of plant growth. In the presence of the dominant pigmentation genes *A1*, *A2* and *C2*, *R1-nj* conditions deep pigmentation of the aleurone layer (endosperm tissue) in the crown and scutellum (embryo tissue) of the kernel. Pigmentation varies in degree and intensity depending on the genetic background of the donor genotype (Geiger and Gordillo, 2009). When the unpigmented source germplasm is pollinated with inducers which carry dominant seed coloration markers, successful fertilization with inducer pollen is visible as a purple colored aleurone (cellular layer of the endosperm) which indicates a fully functional, triploid endosperm

(Fig. 1). The haploids differ from diploids by their colorless scutellum (part of the embryo) indicating a haploid embryo of solely maternal origin.

Greenblatt and Bock (1967) were the first to use the red crown mutant as a selectable marker in haploid induction experiments. They reported that to be effective, the donor parent has to have colorless seeds and the inducer line needs to be homozygous for *R1-nj* and dominant pigmentation genes. The use of haploid inducers with anthocyanin genes *B1(Booster1)* and *Pl1* (*Purple1*) that result in sunlight-independent purple pigmentation in the plant tissue (coleoptiles 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 coleoptiles 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 (Prasanna, 2012).

In addition, Rotarenco *et al.,* (2007) proposed a method of identifying haploid kernels based on kernel oil content using nuclear magnetic resonance. They observed that F1 kernels with haploid embryos had lower oil concentration than those with diploid embryos. Li *et al.,* (2009) developed an inducer line CAUHOI derived from stock6 that allows identification of haploids based on both lack of *R1-nj* conferred scutellum coloration as well as low embryo oil content. Jones *et al.* (2012) explored the utility of Near Infrared Reflectance (NIR) transmission spectroscopy to differentiate haploids from hybrid maize kernels after the maternal haploid induction. Haploid plants can be distinguished from diploid plants by their growth characteristics like erect leaves, poor vigor and sterility. Distinguishing haploids from diploids at seed level is critical for adapting DH technology on a commercial scale since it saves on costs involved in chromosome doubling, green house, field space and labor involved (Prasanna *et al.,* 2012).

Chromosome Doubling

The majority of haploid plants are sterile due to disrupted gamete formation (Tang *et al.,* 2009). Hence, duplication of the haploid chromosomes is necessary to facilitate self-pollination for seed increase and maintenance of the genotype. In maize, the most common chromosome doubling agent, colchicine, is an alkaloid obtained from meadow saffron (*Colchicum autumnale* L.) and it acts as a mitotic inhibitor. Mitosis is the process of nucleus division in somatic cells: after DNA replication, the microtubules pull the duplicated chromatids toward the two poles and the cell divides into two daughter cells. Colchicine disrupts mitosis by binding to tubulin. In this way the formation of microtubules and the polar migration of chromosomes is inhibited and the result is single cells with duplicated chromosome number (Wan *et al.,* 1989).

The procedures developed for colchicines treatment by Deimling *et al.,* (1997) include, germination of putative haploids at 26°C for approximately three days, Preparation of seedlings (reducing the root and clipping the tip coleoptiles), immersion of seedlings in the Colchicine solution (0.06% colchicine, 0.5% dimethyl sulfoxide) for 12 hours at room temperature in a dark room. After the colchicine treatment, the seedlings are carefully washed in water and subsequently grown in the greenhouse to the 5- to 6-leaf stage (during the first days under high humidity). Thereafter, the treated plants are transferred to the field (Geiger and Gordillo, 2009).

Several colchicine-based protocols suitable for large-scale chromosome duplication have been developed for in vivo (Deimling *et al.,* 1997) and in vitro (Wan *et al.,* 1989; Barnab´as *et al.,* 1999) production of maize DH lines. Alternative methods include treatment of haploids with nitrous oxide or with herbicides having anti-microtubule effects (Wan et al. 1991). However, regardless of the method, artificial chromosome doubling of maize haploids is costly, requires special facilities, and/or involves noxious substances.

Applications of Doubled Haploids in Maize Breeding

Development of Homozygous lines

Through the use of DH technology, completely homozygous plants can be obtained in two generations, compared with the conventional methods were multiple generations of self-pollination are required to obtain a partially homozygous plant (Melchinger *et al.,* 2005; Rober *et al.,* 2005; Rotarenco *et al.,* 2010; Seitz, 2005). Rober *et al.,* (2005) reported DH lines are absolutely homozygous and uniform; they ideally meet the requirements for being protected by plant variety rights. They also demonstrated the usage of DH lines allows starting their commercialization several seasons earlier than in conventional breeding. The efficiency of

selection for qualitative and quantitative characters is increased since the recessive alleles are expressed due to the complete homozygousity.

DH in Recurrent Selection Scheme

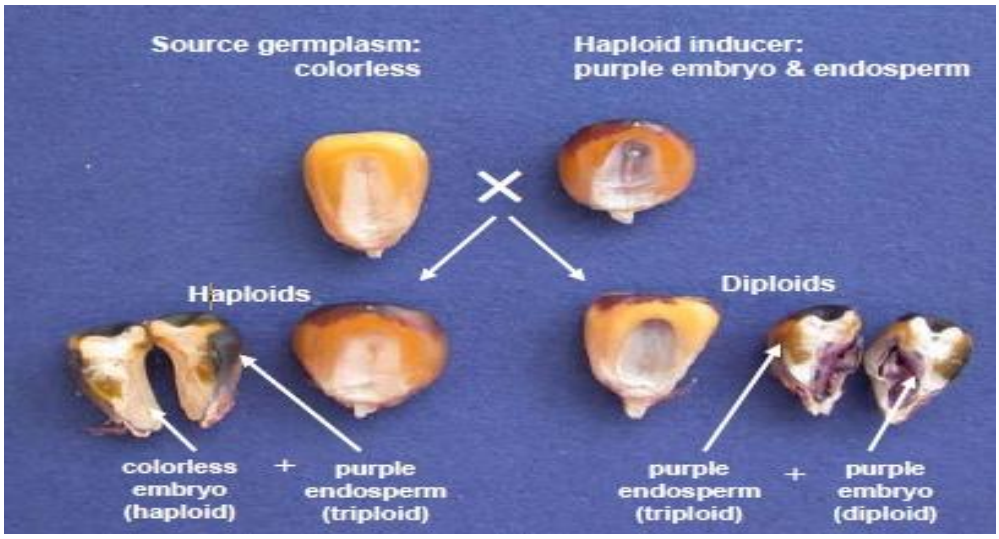
Recurrent selection (RS) constitutes an integral part of hybrid breeding (Hallauer and Miranda, 1988). As long as a breeding population is continuously improved by RS, yield trials for RS can be used as "early testing" trials for hybrid development. Hence, the best lines can be extracted from each cycle of selection for building up hybrids that are superior to those from previous cycles (Suwantaradon and Eberhart, 1974). In hybrid breeding, the aim of RS is to improve the general combining ability (GCA) of a heterotic group with respect to one or several other groups, so called "opposite" pool(s). In this regard, RS based on doubled haploid (DH) lines can be highly effective. Important advantages of using DH lines are a maximum genetic variance between test units and an increased precision in estimating the genotypic value of DH lines and their testcrosses (Rober *et al.*, 2005).

Doubled haploids can be used in a recurrent selection scheme, were after multiple cycles of crossing, DH production and selection, an improvement of the population is expected due to recombination and selection (Bouchez *et al.*, 2000; Gallais, 2009).

Induction of Mutation

Another use of DH technology would be in mutation breeding. The homozygosity enables the fixation of mutations in the first generation after mutagenic treatment (Murovec *et al.*, 2012). Alternatively to the DH technology, pure haploid plants can be used for breeding and research purposes. This is a possibility caused by their ability to produce normal kernels after pollination with the pollen from diploid plants (Chalyk and Rotarenco, 2001; Rotarenco *et al.*, 2012). Maize haploids are good source of materials for mutation study. It is possible to use haploids to estimate spontaneous mutation rate of a specific gene locus. Since haploids only carry a single genome the estimation of mutation rate should be more accurate and straight forward. In microspore culture it is very effective to generate mutants by treating microspores with chemical mutagenesis at uni-nucleate stage, and this will generate pure elite mutant inbred lines (Szarejko, 2003). Another application of the DH method is forward breeding to create new homozygous mutant lines in place of backcross conversion.

Fig.1 Haploid identification, kernel marker R1-nj



Genetic Enrichment

Studies show that recycling DH lines can quickly improve haploid frequency and fertility restoration (Chase, 1951; Liu and Song, 2000). According to Chase (1951) the original stiff stalk synthetic materials yielded 0.13% haploids and the haploid derived DHs yielded 0.43% haploids. The haploid fertility restoration of original stiff stalk synthetic was 9.4% and the DH derived haploids have increased frequency to 33%. It appears that selection favors genetic or germplasm enrichment for production of haploids and fertility

restoration. If that is the case, the germplasm enrichment for yield, general vigor and agronomy of maize plant can be achieved by applying random mating of high yielding DH lines as source of materials for next cycle of haploid selection. Recycling of selected DH lines through recurrent selection or any other breeding scheme is a fast and powerful way to achieve genetic enrichment of inbred lines carrying more favorable alleles for yield, pest resistance, stress tolerance and general agronomic traits (Bouchez and Gallains, 2000; Chalyk and Rotarenco, 2001)

Integration of DH Technology with Marker Assisted Selection (MAS)

In recent years, 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). DH technology can be used in combination with marker assisted selection (MAS) in maize breeding. Application of MAS and DNA fingerprinting together with DHs should be (1) to select parents with complementary genotypes to form crosses for use in deriving DH lines (Prasanna *et al.*, 2010). (2) Use of MAS and DHs could be in recurrent selection projects. Bouchez and Gallais (2000) demonstrated with simulations that use of DH lines will theoretically enhance the efficiency of recurrent selection schemes for traits with low heritability; MAS could be cheaper, faster or more effective than phenotyping DH lines to select parents for subsequent cycles of recurrent selection projects.

A third application in which DH and MAS complement each other is to derive DH lines from bi-parental crosses when the objective is to obtain inbred lines genetically similar to either parent of the cross (Smith *et al.*, 2008) or to identify recombinants at or flanking specific loci. Finally, the most widespread combined use of DH and MAS is probably for genetic studies such as bulked segregant analysis and developing genetic maps (Chang and Coe, 2009; Forster and Thomas, 2005). Because DHs offer a fast way to obtain homozygous lines, they can save time and increase the efficiency of projects designed to identify or map marker-trait associations, leading to potential use of markers in MAS breeding projects (Prasanna *et al.*, 2010).

To meet the rapidly increasing demand for maize product adopting the DH technology in maize breeding plays a crucial role. The DH technology shortens the breeding cycle significantly by rapid development of completely homozygous lines in 2-3 generations, instead of conventional inbred line development process which takes at least 6-8 generations to derive lines ~99% homozygosity. DH genotype is formed when haploid *(n)* cells undergo either artificial or spontaneous chromosome doubling producing two gene sets which are exactly identical. DHs can be produced artificially through *in vivo* or *in vitro* technique. The DH technology in maize breeding, based on *invivo* haploid induction, is recognized worldwide as an important means for enhancing breeding efficiency. The utility of DHs in homozygous lines development, recurrent selection, induction of mutation, genetic enrichment, and integrity with other technologies make them the best material for genetic improvement and genetic studies of maize.

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