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Invivo double haploid technology in maize: Advances, methods, and application in breeding

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ABSTRACT

Double haploid (DH) technology is an efficient approach for the development of 100% homozygous inbred lines in just two generations, it increases the breeding efficiency and genetic gain in maize crop. It is an emerging technique in developing countries such as India, whereas, it has become an integral part of many commercial maize breeding programs in developed countries such as Mexico. This technique offers economic and genetic benefits over the traditional method of inbred line development. This review comprehensively explains the pathway for invivo induction of maternal haploids with brief introduction about other methods of haploid induction being used in maize breeding, factors affecting, challenges and future perspectives for DH technology. Furthermore, it discusses the integration of DH technology with modern breeding tools like genomic selection and CRISPR/Cas9 enhancing the precision and accelerating the development of superior maize cultivars. Automation holds great promise to further reduce the cost and time in haploid identification. The major challenge faced during invivo haploid induction is low haploid induction rate of inducer lines and difficulty in identifying haploid kernels while contributions made have increased the HIR by manipulating the stock -6 to produce lines with high HIR and advanced screening method such as cytometry, fluorescence microscopy, SNPs have been developed for accurate haploid detection. Increasing success rates in chromosome doubling protocols and/or reducing environmental and human toxicity of chromosome doubling protocols, research on genetic improvement in spontaneous chromosome doubling, the potential to greatly reduce the production costs per DH line.

Keywords: *Maize. double haploid, maternal haploids, haploid induction rate, Cen H3, Ig gene, Stock-6 and colchicine*

Introduction

Maize (*Zea mays L.) aka* queen of cereals is one the most significant crops in the world. Along with rice (*Oryza sativa*) and wheat (*Triticum aestivum*) maize is a staple food crop for most of the people in underdeveloped regions of Africa, Asia and America (Dowswell *et al.,* 1996). It is the most versatile emerging crop with respect to its adaptability, types and uses. This crop can withstand a wider range of agroclimatic conditions from tropical to temperate regions, from sea level to an altitude of 2500 mts. More than 170 countries are currently producing 1210 million tons of maize collectively from an area of 205 million ha with an average productivity of 5878 kg/ha (FAOSTAT, 2021). In 2021-22, India produced 33.51 million tonnes of maize on 10.04 million hectares; in *Kharif* 2022–23, however, 23.10 million tonnes (1st advance estimates) of maize were produced on 9.67 million hectares (Reddy, 2022).

Globally, maize production rose from 558 million tonnes in 1995 to 900 million tonnes in 2020 (Erenstein *et al.,* 2022). To feed the projected world's 9.7 billion people by 2050, a 70% increase in total food production is needed. To achieve this goal, hybrids that exploits heterosis to their fullest and the success of modern maize hybrids depends on the breeding of elite parental inbred lines (Duvick, 2005). Two important strategies that can satisfy the demand for maize and its derivati6ves are resource-conservation farming practices and improved crop varieties that outyield other varieties/hybrids with the same or fewer inputs. Traditional methods of maize breeding are labor-intensive and time-consuming, which hinders the development of improved cultivars. To develop sound hybrids, a very common practice is followed by the breeders *i.e.* cross between two distantly related homozygous parental inbred lines (Chiakam *et al.,* 2019). Nowadays, there are two main approaches to develop pure lines: the conventional approach and double haploid breeding. The conventional breeding approach involves recurrent selfing of plants upto six to eight generations, or three to five years when two seasons per year may be possible whereas, the double haploid (DH) technique quickly produces pure lines in just two generations (Geiger, 2009). This technology efficiently incorporates desired agronomic features into elite germplasm to increase genetic gain in crops like maize (Zea mays L.). This review paper provides comprehensive information about the *invivo* double haploid technology in maize (*Zea mays* L.) covering its history, status, methods of development, applications, challenges and future perpectives.

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Why use DH technology in maize?

The conventional method is a time-consuming and labour-intensive approach as it requires recurrent selfing for 6-7 generations to achieve 99% of homozygosity (Chaikam *et al.,* 2019 a). Whereas, DH technology significantly shortens the breeding cycle by the development of completely homozygous lines in just two generations. In the traditional inbred line development method skilled personnel is needed to avoid contamination during self-pollination, adding to the overall cost and complexity, negligence in the process can lead to the risk of genetic drift and loss of genetic diversity reducing the breeding population's resilience to biotic and abiotic stresses and limiting

future improvement. Thus, compared to the traditional method of inbred line development, the use of DH lines in maize breeding provides several genetic, economic, and logistical benefits. Breeders can accelerate their rate of genetic gain (the rate at which a crop's genetic potential yields over time-by reducing time and increasing selection precision) with the use of a double haploid inbred line development approach in maize (Xu *et al.,* 2017). DH being fully homozygous meets DUS (Distinctness, Uniformity, and Stability) requirements, on the other hand, residual heterozygosity in traditional inbred lines might occasionally delay the plant variety registration procedure (Robert *et al.,* 2005). DH technology enables greater efficiency and precision of selection (Rober *et al.,* 2005; Geiger and Gordillo, 2009), especially when used in combination with molecular markers and year-round nurseries. By enabling rapid pyramiding of favourable alleles for polygenic traits DH technology speeds up the development of new hybrid products impacting stress tolerance in maize (Rober *et al.,* 2005). It also provides opportunities for genetic engineering, functional genomics, molecular cytogenetics, marker-trait association studies, and marker-based gene introgression (Forster *et al.,* 2007).

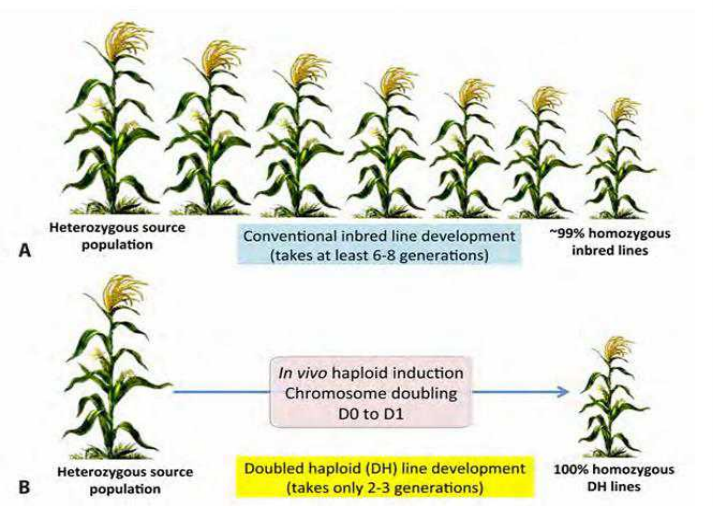

Figure 1: Number of generations to reach genetic purity, Prasanna et al. (2012)

Different methods of DH inbred line production in maize

Both *invitro* and *invivo* method have been used to induce haploids and thereafter, chromosome doubling to produce double haploids in maize.

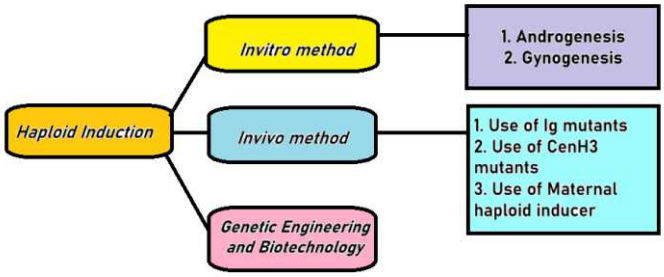

Figure 2: Methods of Double haploid inbred line production in maize

***In vitro* microspore culture (Androgenesis):** Anther culture is a useful tool for creating homozygous maize in one generation only (Barnabas and Obert, 2004).

This technique allows the development of a large number of microspore-derived calli or embryos capable of regenerating viable, fertile DH plants across various genotypes (Barnabas *et al.,* 1999). However, the application of this technique is limited as it is largely dependent on the androgenic responses of the genotype and on the frequency of induced/spontaneous chromosome double in plants originating from microspore. (Geiger *et al.,* 2009). Further, compared to wheat and barley maize shows low recovery of haploid embryo formation from androgenesis (Rober *et al.,* 2005). The protocol for conducting androgensis in maize was described by Zheng *et al.* (2003).

Gynogenesis: Gynogenesis is the process of developing haploid plants from unfertilized ovules/ovaries. In vitro gynogenesis in maize was reported by Ao *et al.* (1982), when obtained 2 % haploid plant by culturing unpollinated ovaries on MS and N_6 medium. The protocol for gynogenesis in maize was detailed and described by Tang *et al.* (2006). The frequency of regeneration of plants from female gametophytes is higher than from male gametophytes (Bugara and Rusina, 1989). However, maize gynogenesis efficiency is influenced by the growing season (Tang *et al.,* 2006). Haploids can be obtained both from the pollinated and unpollinated ovaries (Ao *et al.,* 1982; Alatortseva and Tyrnov, 2001). Truong-Andre and Demarly (1984) reported successful in-vitro gynogenesis when unpollinated ovaries of hybrid maize directly converted into haploid plantlets without the formation of callus.

However, both *invitro* androgenesis and gynogenesis for haploid induction require precise and often complex tissue culture techniques, and need specialized skills and equipment which may not be feasible in all breeding programs thus, limiting their usage. These methods are rarely used for haploid plants development in maize due to their high dependency on the parental genotype, high possibility of mutations in the culture process, and prolonged duration (Chaikam *et al.,* 2012).

***Invivo* methods**

Natural haploid induction by indeterminate gametophyte (ig) mutants: *Ig* gene, discovered in the Wisconsin-23 (W23) line and located on chromosome 3 (Kermicle, 1994), disrupts cell division by interfering with the function of the cytoskeleton in mitosis during the multinucleated cell stage post meiosis of the embryo sac (Kermicle 1969; Enaleeva *et al.,* 1995). Haploids are produced when *ig* mutants are crossed with common inbred lines, impacting paternal haploid induction. The *ig1* mutant eliminates its chromosomes, resulting in a paternal wild-type haploid that contains the *ig1* mutant cytoplasm. (Kermicle, 1969; Geiger, 2009). Kindiger *et al.,* 1993 elevated the frequency of haploid generation upto 8% by the development of a 3(ig) 3(ig) B-3Ld (Ig) tertiary trisomic stock in an improved modified Wisconsin-23 line's background. However, due to low haploid recovery, this gene is less attractive for commercial-scale haploid induction in maize (Geiger, 2009). Also, homozygous *ig* mutants show sterility in some genetic backgrounds of maize which makes their usage limited (Evans, 2007). Paternal haploid inducers transmit cytoplasmic genes to the haploid embryos which may or may not be desirable, thus have limited use (Kelliher *et al.,* 2017; Liu *et al.,* 2017; Gilles *et al.,* 2017).

CenH3- mediated haploid induction: When a line with small or defective centromeres crossed with a normal line having large or normal centromeres, the smaller or defective centromeres will be selectively degraded, resulting in a loss of

chromosomes in the parent having a small centromere (Zhang and Dawe, 2012 and Wang and Dawe, 2018). Wu *et al.* (2017) identified the *CenH3* gene's role in centromere-mediated chromosome elimination, *CenH3* gene was first identified and isolated in Arabidopsis and barley by Ravi and Chan (2010). Kelliher *et al.* (2016) showed that CenH3-tail swap influence maternal haploid induction rate. Haploid progeny can be generated in both ways either crossing wild type plant as male/female to +/cenH3 (Wang *et al.,* 2021).

Integrating *invivo* haploid induction methods such as manipulating stock6- derived inducer lines by overexpressing maize cenH3 could significantly enhance the maternal haploid induction rate (HIR). Substituting a tail-altered CenH3 for the full-length CenH3 in the tagged expression cassette, resulted in a maternal HIR of up to 16.3% (Meng *et al.,* 2022; Khammona *et al.,* 2024). Cyto-swapping in maize *via* haploid induction with cenH3 mutant provides a faster and cost-effective method to convert commercial lines to cytoplasmic male sterile compared to conventional trait introgression (Bortiri *et al.,* 2024).

Invivo maternal haploid induction-based DH development

Invivo maternal haploid induction method are comparatively easier than *invitro* methods with inducer genetic backgrounds, facilitated by integrating an anthocyanin color marker to permit easy identification of haploids both at seed and seedling phases (Coe *et al.,* 1959; Coe and Sarkar, 1964; Nanda and Chase, 1966 and Greenblatt and Bock, 1967).

Haploid inducers are specialized genetic stocks when crossed with normal (diploid) maize results in a certain fraction of haploid kernels due to anomalous fertilization. Several haploid inducer lines have been developed by using Stock6 as a founder. Such as RWS, RWK-76, UH400, and UH600 (University of Hohenheim, Germany), the PHI series (Procera, Romania), CAU5 (China Agricultural University, China), the BHI series (Iowa State University, USA), and the TAIL series (Tropical Adapted Inducer Lines, *Cimmyt*) (Geiger and Gordillo, 2009; Rotarenco *et al.,* 2010; Prigge *et al.,* 2012b; Xu *et al.,* 2013; Chaikam *et al.,* 2018; Trentin *et al.,* 2020; Trentin *et al.,* 2023; Chen *et al.,* 202). When inducers are use as pollen source parents it is called as maternal haploid inducer yielding haploid embryos having maternal genome only. The seed obtained from haploid induction crosses must be screened for haploids and the most used method for identifying haploid seeds is the *R1-nj* gene (Navajo) (Liu *et al.,* 2016). This *R1-nj* gene imparts anthocyanin pigmentation to the haploid seeds on the kernel crown region which will appear red/purple, while the embryo will be uncolored (Liu *et al.,* 2016). This method is beneficial as it speeds up the breeding programe and allows large scale haploid induction for double haploid line production in maize (Lie *et al.,* 2015; Melchinger *et al.,* 2013).

Genetic Engineering and Biotechnology

The genome editing tool *CRISPR/CAS9* tool can significantly enhance the haploid induction rate (HIR) in maize. Advanced genome editing methods such as genome editing with haploid induction (HI-edit) and haploid inducer-mediated genome editing system (IMGE) have been developed to increase the frequency of haploid induction in maize plants (Kelliher *et al.,* 2019; Wang *et al.,* 2019). These techniques have shown considerable promise in improving breeding processes. The Haploid-Inducer Mediated Genome Editing (IMGE) approach could accelerate the maize breeding by rapid development of genome-edited genetically pure doubled haploid lines, which possess desirable traits in elite genetic background (Wang *et al.,* 2019). A notable achievement reported by Wang *et al.,* (2019) involved the successful generation of genome-edited haploids for ZmLG1 and UB2 within the B73 genetic background using the CAU5 haploid inducer line engineered to carry the CRISPR/Cas9 cassette. This demonstrates the practical application and effectiveness of CRISPER/Cas9 in improving haploid induction rates. In these methods, the CRISPR/Cas machinery is integrated into a haploid inducer line, this modified inducer line is then use to pollinate maternal elite lines leading to the elimination of paternal genome for the haploid production. Inducing specific mutations in genes namely *MATRILINEAL* (MTL) (Kelliher *et al.,* 2017), *Zea mays* Phospholipase A1 (*ZmPLA1*) (Liu *et al.,* 2017) and *NOT LIKE DAD* (NLD) (Gilles *et al.,* 2017) increases the haploid induction rate of inducer lines by 2 to 10%. These targeted genetic modifications are instrumental in refining the efficiency of haploid production, thereby supporting more effective and rapid breeding strategies.

History of _invivo_ maternal haploid induction-based DH development

Chase (1947) pioneered double haploid maize inbreeding using spontaneous parthenogenesis (Randolph, 1932; Chase, 1947). Due to the extremely low frequency of spontaneous haploid induction (0.1%) this technique limited its commercial viability (Chase, 1951). In 1959, Coe detected a higher haploid induction rate of upto 2.4%, induction crosses developed by using "inbred lines stock 6" inducer line revolutionized *invivo* haploid induction. Stock-6 inbred lines are described as 'line of maize with high haploid frequency' Coe (1959), became the progenitor for further inducer lines development across the globe. For example, inducer line WS14 developed by crossing lines W23ig and Stock 6 showed a haploid induction rate upto 3–5% (Lashermes and Beckert,1988). In the last 15 to 20 years, more advanced inducers with a high HIR of 8–10% have been developed using the Stock 6 genotype, enabling the large-scale production of maize DH lines (Geiger and Gordillo, 2009).

In India, Dr. K R Sarkar carried out important research on haploid induction at IARI, New Delhi and achieved a haploid induction frequency of approximately 6% (Sarkar *et al.,* 1972). Dekalb 640 maize hybrid created in the 1950s, was the first widely planted commercial hybrid developed from a DH programme through a double cross of three DH and one conventional inbred parent line, (Liu *et al.,* 2016).

Modern inducers that have been improved include the PHI series (Procera, Romania), CAU5 (China Agricultural University, China), RWS, RWK-76, UH400, and UH600 (University of Hohenheim, Germany), the BHI series (Iowa State University, USA), and the TAIL series (Tropical Adapted Inducer Lines, *Cimmyt*). First-generation tropically adapted inducer lines (TAILS) (including TAIL 5, and TAIL 7 to TAIL 9) were developed by *Cimmyt* and Hohenheim University, these inducer lines have an HIR of about 5.5-11% (Prasanna *et al.,* 2012, Prigge *et al.,* 2012a). The second-generation TAILS (CIM2GTAILS with HIR 9-14%) were developed by *Cimmyt* exclusively (Prasanna *et al.,* 2012, Chaikam *et al.,* 2018, Chaikam *et al.,* 2019a).

Status across the globe and in India

The specific status of DH technology in maize breeding can vary across different regions and countries, including India.

Globally, DH technology has been increasingly adopted by maize breeders and seed companies to accelerate the development of

new maize varieties/hybrids having desired traits such as higher yield, disease resistance, and tolerance to abiotic stresses. It is widely adopted in Europe, North America, and China maize breeding programmes (Chaikam *et al.* 2019b; Nzamu 2018; Molenaar and Melchinger, 2019). The technology is gaining popularity in sub-Saharan Africa following the establishment of the Maize Doubled Haploid Facility in Kiboko Kenya by *Cimmyt* in partnership with Kenya Agricultural and Livestock Research Organization (KALRO) (Chidzanga *et al.,* 2019). *Cimmyt* has established a DH line production facility for Latin America as well as an experimental station of *Cimmyt* in Agua Fria, Mexico. A similar facility is also in operation for Latin America at *Cimmyt*'s experimental station in Agua Fría, Mexico (anonymous, 2017). Development and release of improved maize hybrids with DH lines as parents have been reported in Africa (Beyene *et al.,* 2017; Chaikam *et al.,* 2018).

In India, DH technology in maize breeding has also gained traction in recent years, however, the extent of adoption and the specific advancements of DH technology in India would require more current, region-specific information. Major maize breeding companies use using DH lines in their programmes, either sourced from their parent companies/associates overseas or developed in their Indian programmes. However, the Indian maize research program has been slow to pursue and capitalize on the initial work resulting lagged in adopting DH technology and reaping its benefits. Barring a few reports on some elementary work on double haploid there is hardly anything substantial as far as large-scale production of DH lines and release of DH-based hybrids is concerned (Roop Kamal 2017; Khulbe *et al.,* 2019, 2020; Showkath Babu *et al.,* 2020, Gupta, 2022). Limited large-scale production and release of DH-based hybrids attributed to technology knowledge and experience, funding and fundamental infrastructure deficiencies. The process of producing DH is resource-intensive and necessitates certain fundamental infrastructure, preventing maize breeders from utilizing DH technology in their breeding operations. On December 3, 2021 *Cimmyt* with UAS Bangalore inaugurated a state-of-the-art maize double haploid facility in Kunigal, Karnataka (Anonymous, 2021). In India, among the public sector institutions, work on DH is being conducted at ICAR-VPKAS (Almora), CSKHPKV HAREC (Bajaura), PAU (Ludhiana) and ICAR-IARI (New Delhi). DH lines have been produced at these centers and are being used in breeding programmes all over the country. At the ICAR level, initiatives are underway to include DH breeding into the national maize improvement programme.

Pathway for *Invivo* maternal haploid induction- based DH development (Khulbe and Patnayak, 2020)

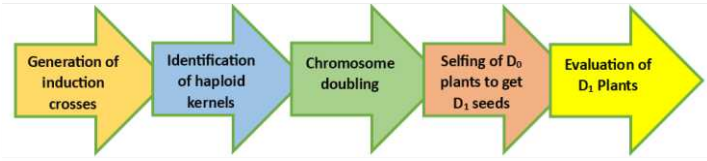

Figure 3: Pathway of double haploid (DH) development using inducer line

A. Generation of induction crosses: A cross is conducted between haploid inducer line (pollen parent) (HIL) and source germplasm termed as induction cross. The number of plants required to be raised for a particular induction cross depends on the targeted number of DH lines to be derived and the desired haploid seeds quantity. The ratio of source germplasm plants and HIL plants determined by the inducer lines' pollen production ability. The planting layout of the source germplasm block and HIL block are kept separate but close ensuring cross-pollination using the hand pollination method and avoiding self-pollination. To obtain an optimal number of haploid seeds, a haploid inducer with a and high haploid induction rate should be preferred. Each induction cross will be harvested separately, labelled correctly to avoid accidental intermixing. The ears will be adequately dried and safely stored in separate labelled bags until haploid seed sorting (Prasanna *et al.,* 2012 and Khulbe *et al.,* 2019).

B. Identification of haploid seed: The induction cross seed is a mixture of F_1 seed, haploid seed and self-/outcross-seed/pigmentation-inhibited seed. The haploid seed is separated from the mixture based on seed and/or seedling traits.

1. Sorting of induction cross can be done in two ways:

- Shelling the ears of induction cross altogether and bulking them. A handful from the bulk may be drawn each time to look for and separate the haploid seed from it, by looking at the pigmentation on the kernel crown and scutellum.
- The seeds are removed using the other hand one by one while rotating the cob clockwise or anti-clockwise. This allows the crown region as well as the embryo region of the seed clearly visible while it is still embedded in the ear.

2. Identification of haploid seed/seedling

- Based on seeds: Haploid seeds can be separated out based on the expression of anthocyanin pigmentation on kernel endosperm and scutellum conditioned by R1-nj marker (Nanda and Chase 1966; Greenblatt and Bock 1967).

Based on seedling: A haploid inducer with red/purple root marker is used for facilitating the separation of haploid and diploid seedlings (Rotarenco *et al.,* 2010; Chaikam *et al.,* 2016; Chaikam *et al.,* 2019b). Seedling traits such as radical length, coleoptile length, seminal lateral roots and root hairs have also been used for haploid classification in germplasm where the R1-nj method cannot be employed (Chaikam *et al.,* 2017).

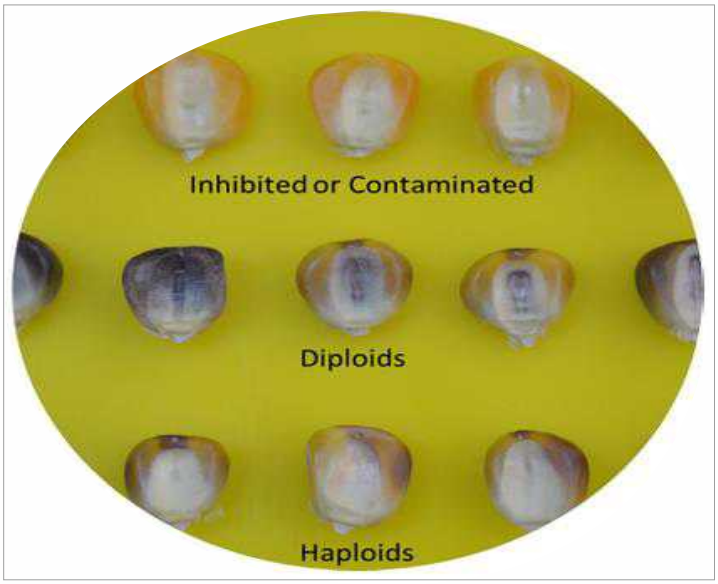

Figure 4: Classification of seed resulting from haploid induction crosses into different categories, Chaikam et al., 2019a

A. Chromosome doubling: The spontaneous chromosomal doubling occurs at a very low frequency and, thus, necessitates the chemically induced doubling for double haploid generation. Mitotic inhibitors are the most utilized chemicals for this purpose. Colchicine is a commonly used chemical for chromosome doubling though other agents like nitrous oxide, trifluralin, amiprophos-methyl (APM), pronamide, and oryzalin are also effective. Among them N_2O is relatively safe and has no health effects but it requires initial investment (Chaikam *et al.,* 2019 a).

The colchicine dose and treatment duration vary in different protocols. In this method, the haploid seeds are allowed to germinate in paper towels for 72 hours at 25-28ºC. The root and shoot tissues of the seedlings are cut upto 2 cm and 1 cm from the tip, respectively, and the cut seedlings are immersed in a solution of 0.04% colchicine + 0.5% DMSO for 12 hours. (Chaikam and Mahuku, 2012b).

B. Transfer of colchicine-treated seedlings into cup trays (D_0 plants): In the morning, colchicine solution from the treated seedling is drained in the colchicine disposal can be followed by two time wash with plain water to remove colchicine from their surface. The treated seedling is then put into the potting medium (a mixture of cocopeat and vermicompost in 1:3 ratio by weight).

C. Transplanting of D_0 from cups into the field: Transplanting of maize seedlings is carried out at the 3-4 leaf stage. After the translanting of D_0 seedlings into the field, proper management practices regarding nutritional requirement, insect -pest, and disease control will be followed to maintain healthy state of the haploid plants. Diploid plants that can be readily identified will be removed at the vegetative stage itself. However, for plants that exhibit intermediate features, their rouging may be delayed until the tasseling stage.

D. Selfing of fertile D_0 plants: The colchicine treatments render a small proportion of the D_0 plants fertile. These fertile plants are self-pollinated to obtain selfed seed. Selfing of D_0 plants is carried out in the same manner as in the case of normal maize inbred lines. Haploids are sensitive to postemergence herbicides compared with normal maize; therefore, mulching or other cultural practices may be used for weed control. Haploid tassels show significant variation in fertility ranging from a few anthers to a whole tassel capable of producing pollens; therefore, tassels should be monitored regularly for extruded anthers. Each D_0 plant with fertile tassels should be self-pollinated two to three times on consecutive days to ensure good seed setting. Ears from D_0 plants are harvested at physiological maturity and the seed of each harvested ears is referred to as the D1 generation (completely homozygous for the set of genes).

E. Harvesting of D_1 ears: The self-pollinated plants in the D_0 nursery are being harvested and the selfed seed obtained on the D_0 plant is the D_1 seed.

F. Raising and handling of D_1 nursery: Since all the plants in the D_1 nursery are essentially diploids, the same package of practices as recommended for raising conventional inbred lines may be followed.

G. Evaluation of DH lines: Selfed-seeds obtained from the fertile D_0 plants i.e. D_1 seed, are subsequently raised for maintenance and evaluation.

For identifying contaminated lines/off-type plant molecular markers (foreground and background markers) can be used to screen the DH lines.

Factors influencing the double haploid production

a. Genetic factors: Maternal haploid induction is highly influenced by the nature and type of germplasm used as the female donor parent (Eder and Chalyk, 2002). Haploid inducers of temperate origin are ineffective for tropical or subtropical germplasm, highlighting the importance of matching the inducer and source population origin (Ribeiro *et al.,* 2018). The efficiency of haploid induction largely depends on the HIR of inducer lines (Chaikam *et al.,* 2019a). Additionally, R1-nj markers for haploid seed sorting is a relatively inefficient approach due to variable pigment expression under different conditions, incomplete penetrance, and inhibition of expression by inhibitor genes. Therefore, Liu *et al.,* 2012 proposed a high-throughput NMR system of screening for the rapid discrimination of haploid seed from diploids based based on kernel oil content by pollinating source population with high oil inducer lines (Melchinger *et al.,* 2013; Wang *et al.,* 2016). Furthermore, variations in maternal genotypes affects the efficiency and frequency of haploid embryo formation (Fuente *et al.,* 2018; Liu *et al.,* 2020). The genetic factors collectively determine the success rate and efficiency of haploid induction, emphasizing the need for careful selection of both the inducer lines and source germplasm.

b. Environmental factors: Environmental factors play a crucial role in the success of haploid induction in maize, with genetic and environmental factors such as temperature and light intensity significantly influencing the haploid induction rate. Climatic factors such as temperature and humidity influence the HIR in maize, especially during pollination. Kebede *et al.,* 2011 observed that the HIR was 7.37% during the winter season, which was notably higher than the 6.11% observed during the summer season in Mexico, demonstrating the importance of seasonal conditions. Maintaining optimal agronomic conditions is essential for maintaining the haploid fertility. Stress often leads to male sterility and under severe conditions female sterility has also been observed (Vanous *et al.,* 2017). Additionally, the expression of the *Navajo* gene integrated with B1 (booster) and Pl1 genes responsible for imparting purple coloration to above-ground tissues are effected by plant growth conditions, heat stress, sunlight intensity, temperature and many other environmental factors that can influence the efficacy of B1 and Pl1 genes, and indicating the sensitivity of haploid induction processes (Rotarenco *et al.,* 2010).

Applications of DH in maize breeding

A. Accelerated Breeding: The DH technology significantly speeds up the breeding process of pure inbred lines development compared to a conventional method. This is an advanced breeding approach to develop entirely homozygous plants in two generations whereas the conventional method takes 6-7 generations of recurrent self-pollination (Seitz *et al.,* 2005; Rotarenco *et al.,* 2010). Since DH lines are completely homozygous and homogenous, they offer greater genetic uniformity and stability compared to traditional lines which makes them good candidates for plant variety protection. It allows breeders to identify and select lines with superior agronomic traits, disease resistance and other beneficial traits (Bernado *et al.,* 2009).

B. Hybrid Development: The uniformity and genetic stability of DH lines are crucial for producing consistent and reliable hybrids, ensuring the expression of desirable traits across all the plants (Geiger and Gordillo, 2009). Maximum heterosis for yield, resistance to stresses and other agronomic traits can be achieved by using elite and homozygous DH lines (Chaikam *et al.,* 2019).

C. Recurrent selection (RS): Recurrent selection is an important component of hybrid breeding. The goal of recurrent selection to enhance a heterotic group's General Combining Ability (GCA) in comparison to other groups and RS based on DH lines is quite efficient in this regard (Gallias *et al.,* 2007). Using DH lines has several advantages, including a maximum genetic variance across test units and improved precision in evaluating the genotypic value of DH lines and their testcrosses (Rober *et al.,* 2005). Due to recombination and selection, doubled haploids can be used in a recurrent selection system, in which the population is projected to improve after repeated cycles of crossing, DH generation, and selection (Bouchez *et al.,* 2000; Gallais *et al.,* 2009)

D. Marker-assisted selection: Highlight the integration of DH technology with marker-assisted selection to enhance breeding efficiency. DH lines are invaluable for genetic mapping and marker-assisted selection (MAS). Their homozygosity simplifies the identification of genetic markers linked to desirable traits. This facilitates the use of MAS in breeding programs, allowing breeders to select for traits at the seedling stage, thereby accelerating the development of new hybrids (Prasanna, 2012). The goal of combining MAS and DNA fingerprinting with DHs is to select parents with complementary genotypes to form crosses for use in generating DH lines (Parsanna *et al.,* 2010) or finding recombinants at or flanking specified loci. The most common combined usage of DH and MAS is expected to be genetic research such as bulked segregant analysis and constructing genetic maps (Foster *et al.,* 2005; Chang *et al.,* 2009).

E. Mutation breeding: Homozygosity of DH lines allows fixing of mutation in one generation (Murovec *et al.,* 2012). In microspore cultivation, treating microspores with chemical mutagenesis during the uninucleate stage is a particularly effective way to create mutants, resulting in pure elite mutant inbred lines (Szareiko *et al.,* 2003).

F. Cytoplasmic male sterility: Ig1 system of paternal haploid induction is used in maize breeding for the generation of cytoplasmic male sterile lines. pollinated with distinct inbred lines, paternal haploids with CMS are produced. To create a new diploid CMS line, pollen from the maternal inbred must be deposited on one or a few paternal haploids. Three main benefits come from using paternal haploids for cytoplasmic conversions: The new CMS line has 100% of the genomes of the inbred line; (i) only two generations are needed; (ii) chromosome doubling is not necessary (Evans *et al.,* 2007).

Challenges in DH technology

Theoretically, the double haploid (DH) system in maize has the potential to accelerate genetic gains significantly but, faces numerous challenges at each step. The haploid induction rate (HIR) is a quantitatively controlled trait limiting the number of haploid progeny due to the involvement of complex genetic background (Prigge *et al.,* 2011; De La Fuente, 2015).

The nature and type of germplasm used as the female donor parent significantly influence maternal haploid induction (Eder and Chalyk, 2002). In some cases, the maternal haploid induction rate may decrease up to 58% after just one generation of self-pollination (Ribeiro *et al.,* 2018).

Mischaracterization of haploid kernels is another challenge; however, this issue can be mitigated through improved lightening in work spaces, repeated practice, and automation (Vanous *et al.,* 2017). Establishing DH facility at an ideal stress-free and resource-rich location, is very crucial for the recovery of double haploid plants. However, the identification of such locations is a big challenge in the establishment of DH facilities particularly in developing countries (Prasanna *et al.,* 2012; Chaikam *et al.,* 2019a).

Chromosome doubling is primarily reliant on colchicine treatment is a limiting factor especially in developing countries (Kleiber *et al.,* 2012). Despite extensive multi-season studies involving skilled labor and modernized research facilities, chromosome doubling rates remain below 1%. This lower success rate is more pronounced in specialty maize cultivars, such as sweet corn and popcorn, according to observations from the Iowa State University Doubled Haploid Facility and reported by De La Fuente (2015). Amiprophos-methyl, combined with an optimum dosage of pronamide, proved a promising alternative to colchicine in terms of lower toxicity and comparable rate of chromosome doubling in maize (Melchinger *et al.,* 2016). Addressing these challenges, through improved techniques and alternative treatments is essential for the broader deployment and success of DH technology in maize breeding programs.

Future perspectives

To accelerate the development of high-quality hybrids by institutions located in maize-growing tropical/subtropical regions, certain key factors must be considered including adopting effective and safe operational practices, providing adequate training to scientific and technical staff, utilizing Marker-Assisted Selection (MAS), and maintaining year-round nurseries (Prasanna *et al.,* 2012). Utilizing Marker-Assisted Selection (MAS) of crucial loci involved in haploid induction, combined with thorough phenotypic selection, holds the potential to facilitate the development of novel haploid inducers with remarkably high HIR percentages (potentially exceeding 20%). The automation of haploid identification processes, employing diverse phenotypic markers such as high oil content or the R1-nj trait, is swiftly transitioning from concept to reality, with multiple research groups actively developing prototypes. The occurrence of SCD in the male tassel of maize varies from 2.8% to 46%, and in the female tassel, it ranges between 25% to 94% in the context of in vivo haploid induction. Further, the implementation of Spontaneous chromosome doubling (SCD) in maize at a commercial level not only eradicates the necessity for colchicine treatment and shifting but also conserves time, resources, and labour expenses. Moreover, it diminishes potential human health risks and seed alterations associated with colchicine use (De La Fuente, 2015). De La Fuente (2015) further highlighted that the expenditure on line development was trimmed by up to 91% with the utilization of SCD instead of colchicine treatment. The incorporation of embryo rescue techniques in DH protocol can accelerate the line development process by up to 3 months (Barton *et al.,* 2008), indicating the future employment of embryo rescue techniques in DH technology once, the protocol for haploid embryo identification, chromosome doubling and recovery of seedling from embryos become widely available.

Recently, Doubled Haploid (DH) technology has shifted from its exclusive use in recycling elite germplasm to creating inbred lines for hybrid maize breeding. There is a growing trend in utilizing DH technology to explore genetic diversity within maize landraces, aiming to protect genetic resources and introduce new variations to broaden the genetic foundation of elite germplasm (Melchinger et al. 2018; Böhm et al. 2017; Brauner et al. 2019). A more recent advancement known as Haploid Induction-based Genome Editing (HI-Edit) or Haploid Inducer Mediated Genome Editing (IMGE) allows for direct genomic modification of commercial inbred lines. This innovation streamlines the process of incorporating genome-edited traits into superior cultivars by bypassing several time-consuming and expensive steps (Kelliher *et al.,* 2019; Wang *et al.,* 2019). Utilizing transgenic double-fluorescence proteins like eGFP driven by an embryo-specific marker and dsRED driven by an endosperm-specific marker, along with genome editing targeting the MTL gene facilitates both the induction and reliable identification of haploids in numerous crop species (Dong *et al.,* 2018). The integration of such cutting-edge technologies is expected to unlock the full potential of Doubled Haploid (DH) technology in maize and other important crop species soon.

Using genetic engineering techniques such as knockout and RNAi of the ZmPLA1 gene can enhance the rate of haploid induction. For example, the CRISPR/Cas9 technology has been shown to achieve almost 2% haploids, confirming the targeting of the gene GRMZM2G471240 (Kelliher et al. 2017; Liu et al. 2017; Gilles et al. 2017). A single-nucleotide change in ZmDMP (protein-coding sequence of the gene GRMZM2G465053, which encodes a DUF679 domain membrane protein) leads to a 2- to 3-fold increase in the HIR (Zhong *et al.,* 2019). Knockout of ZmDMP triggered haploid induction, with an HIR of 0.1 to 0.3%, and there was a greater ability to increase the HIR by 5- to 6-fold in the presence of mtl/pla/nld (Zhong et al. 2019). The cloning of these two induction genes (ZmPLA1 and Zm DMP) provided important information for studying the molecular mechanism of haploid induction and improving the DH breeding efficiency in maize.

Conclusion

In plant breeding, the ability to produce doubled haploids (DH) in maize is a breakthrough that could speed up genetic gains and improve the creation of hybrid varieties with desired features. DH technology implementation is not without its challenges though. The genetic complexity of haploid induction rates and climate factors like temperature and humidity are important determinants of haploid induction success. The approach of producing DH is further complicated by the impact of maternal germplasm, the efficiency of haploid identification, and the technical difficulties related to chromosomal doubling, specifically the dependence on colchicine. Promising answers to some of these problems can be found in automation advancements, enhanced sorting methods, and alternative chemical agents to colchicine such as amiprophos-methyl in combination with pronamide. However, establishing DH facilities in the ideal places with sustainable resources is still a major challenge, particularly in developing countries. To fully harness the potential of DH technology, continued research and innovation are essential. Improving the efficiency and success rates of DH production will require a multidisciplinary approach, integrating advances in genetics, agronomy, and biotechnology. By addressing these challenges and optimizing the DH process, maize breeders can more effectively develop high-yielding, resilient hybrid varieties, ultimately contributing to global food security and agricultural sustainability.

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