***Review Article***

**Advances in Haploid and Doubled Haploid Technology for Accelerating Breeding Cycles in Crop Improvement**

**Abstract**

Doubled haploid (DH) technology represents a major advancement in plant breeding, offering a rapid and efficient method to produce completely homozygous lines in a single generation. This approach significantly shortens breeding cycles and enhances genetic gain by accelerating the fixation of desirable traits. DH lines have become indispensable in hybrid development, molecular breeding, quantitative trait loci (QTL) mapping, genomic selection, and functional genomics. The technology encompasses both in vivo methods, such as haploid inducer lines and genome elimination, and in vitro methods like anther, microspore, and ovule culture. Key genetic components such as MTL, DMP, and CENH3 play critical roles in haploid induction, and recent advances in CRISPR-Cas systems have enabled simultaneous genome editing and DH line creation. Despite its advantages, DH technology faces limitations including genotype dependency, low efficiency in some crops, technical complexity, cost, and challenges in chromosome doubling and plant regeneration. Moreover, the use of transgenic haploid inducers raises biosafety and regulatory concerns. Integration with modern tools such as speed breeding, artificial intelligence, synthetic biology, and high-throughput phenotyping has improved the scalability and precision of DH systems. Applications now extend beyond major crops to include recalcitrant and orphan species, supporting climate-resilient breeding efforts. Machine learning models are increasingly used to predict haploid induction success, embryo viability, and breeding values from large genomic datasets. Automation in embryo rescue, chromosome doubling, and screening is also enhancing throughput and reducing labour. As the global demand for food and climate-resilient crops intensifies, DH technology continues to be a critical component of next-generation breeding programs. This review presents a comprehensive synthesis of DH methods, molecular mechanisms, applications, challenges, and future prospects aimed at maximising the potential of this technology in crop improvement. Case studies on major crops revealed that in maize, DH technology has become an integral part of commercial breeding. In *Brassica napus*, microspore culture is the preferred DH method due to its high efficiency and scalability. Similarly, in *Capsicum annuum* (pepper), anther culture has been employed to develop DH lines for hybrid seed production and disease resistance breeding. Furthermore, the success of haploid and doubled haploid (DH) technologies often hinges on the genetic background of donor plants. It was emphasised that as global food demand rises, DH technology remains pivotal for delivering resilient, high-yielding cultivars with enhanced genetic gains under changing climatic conditions.

**Keywords:** *Breeding, CRISPR, Genomics, Haploids, Homozygosity, Inducers, Phenotyping*

**I. Introduction**

***A. Background on global food security and the need for rapid crop improvement***

The global demand for food is projected to rise substantially with the increase in population, which is expected to reach 9.7 billion by 2050 (Falcon *et.al.,* 2022). Climate change, declining arable land, and increasing biotic and abiotic stresses have intensified the urgency to develop high-yielding, stress-resilient crop varieties. To ensure food and nutritional security, crop improvement must be accelerated beyond the pace of traditional breeding programs. The traditional breeding techniques used for developing new crop varieties are often time-consuming and cannot keep up with the exponentially rising demand for food production. However, with the advancement of technologies and breakthroughs, researchers and breeders are able to accelerate the advancement of novel varieties (Potts et al., 2023).

Traditional plant breeding, which relies on multiple cycles of selection and recombination, can take 8–12 years to develop a stable, high-performing variety. This long duration delays the delivery of improved cultivars to farmers and constrains the capacity to respond rapidly to emerging challenges such as pest outbreaks and shifting climatic conditions. Rapid breeding tools are essential to shorten generation time and to quickly fix desirable traits in breeding lines, making crop development more responsive and efficient.

***B. Limitations of conventional breeding in terms of time and genetic fixation***

Conventional breeding methods, including pedigree and recurrent selection, are time-consuming and resource-intensive. The process of achieving complete homozygosity through successive selfing may require 6–8 generations, making the breeding process inefficient for rapidly addressing food production needs. The reliance on natural recombination and segregation also leads to genetic drag and linkage disequilibrium, which can limit genetic gain per unit time.

Moreover, the genetic base of breeding populations can narrow over time, reducing the potential for introducing new beneficial traits (Cooper *et.al.,* 2001). In crops with complex or polyploid genomes, such as wheat and canola, these challenges are exacerbated, complicating the fixation of desirable traits and increasing the duration needed to reach true breeding lines. These constraints underline the need for alternative technologies that allow rapid fixation of traits while preserving or enhancing genetic diversity.

***C. Introduction to haploid and doubled haploid (DH) technologies as solutions***

Haploid and doubled haploid (DH) technologies offer a transformative solution to these limitations by enabling the production of completely homozygous lines in a single generation. Haploids possess a single set of chromosomes (n), and upon chromosome doubling—spontaneously or chemically induced—they become doubled haploids (2n), genetically stable and uniform. This technology eliminates the need for repeated selfing and accelerates the breeding process by reducing the time required to achieve genetic fixation from several years to a few months. In a number of basic research investigations as well as practical research, doubled haploids (DH) have proven to be a beneficial tool (Chen et al., 2024).

The efficiency of DH systems has been successfully demonstrated in major cereal crops such as wheat, maize, and barley, as well as in oilseeds and vegetables. In maize, for instance, the DH system has been widely adopted for hybrid seed production due to its ability to rapidly generate homozygous inbred lines, which form the backbone of commercial hybrids. The role of DH in the breeding process is largely determined by the mechanism of reproduction used by the plant. They might be final cultivars in self-pollinated species, or they can be utilised as parental lines in hybrid development or test-crosses in cross-pollinated species (Yali, 2022). The precision and speed of DH technology make it a powerful tool for integrating novel traits, including those introduced via molecular breeding or genome editing.

***D. Objectives and scope of the review***

This review aims to consolidate current advances in haploid and doubled haploid technologies, with an emphasis on their application in accelerating breeding cycles across crop species (Sood *et.al.,* 2015). It explores the methodologies of haploid induction and chromosome doubling, the molecular mechanisms underlying these processes, and the integration of DH systems into modern breeding strategies such as genomic selection and CRISPR-based genome editing. The review also highlights species-specific advancements, evaluates practical challenges and bottlenecks, and discusses future prospects in leveraging DH technology for climate-resilient, high-yielding crop varieties.

**II. Theoretical Background**

***A. Definitions: Haploids, doubled haploids, and their significance***

*Haploids* are plants or cells that possess only one set of chromosomes (n), representing the gametophytic chromosome number (Murovec *et.al.,* 2011). In contrast, *doubled haploids (DHs)* are homozygous individuals developed by doubling the chromosome number of haploids to form diploid (2n) organisms. This transformation from haploid to DH restores fertility and allows stable inheritance in subsequent generations.

The primary significance of haploids and DHs lies in their complete homozygosity, which is otherwise achieved through multiple generations of self-pollination or backcrossing. This single-generation homozygosity drastically reduces breeding time. For instance, in maize, homozygosity achieved through the DH method takes only 1–2 years, compared to 6–8 years through conventional inbreeding.

DH lines are essential for hybrid breeding, genetic mapping, genomic selection, and functional genomics (Fu *et.al.,* 2022). They also serve as valuable tools in reverse genetics and mutagenesis studies due to their genetic stability and uniform expression of phenotypes.

***B. Genetic and cytological basis of haploid induction***

Haploid induction can occur through a variety of cytological mechanisms, including parthenogenesis (development from unfertilized egg cells), androgenesis (development from male gametes), and genome elimination during interspecific or intraspecific crosses. These mechanisms are often triggered by either in vivo or in vitro strategies, depending on the species and method used.

*Androgenesis* typically involves the reprogramming of microspores (immature pollen) into embryogenic pathways under stress treatments (such as cold, starvation, or chemical exposure). This results in haploid embryos derived from male gametophytes.

*Genome elimination*, a mechanism largely explored in cereals such as wheat and maize, is based on the selective loss of chromosomes from one parent (Ishii *et.al.,* 2016). For example, in the wheat × maize system, maize chromosomes are actively eliminated post-fertilisation, resulting in haploid wheat embryos.

***Key genes involved in haploid induction***

* *MTL (MATRILINEAL)* gene in maize is crucial for in vivo maternal haploid induction. Mutations in this pollen-specific phospholipase trigger genome elimination, producing haploids at a frequency of 8–15%.
* *CENH3* (centromeric histone H3) modification or replacement has been shown to induce haploids in Arabidopsis by causing centromere dysfunction and uniparental chromosome loss.
* *DMP (DOMAIN OF UNKNOWN FUNCTION 679 MEMBRANE PROTEIN)* genes have been identified to mediate haploid induction across several dicot species, broadening the applicability of gene-based haploid systems.

***C. Historical milestones in haploid and DH technology development***

By the 1960s, the concept of androgenesis through anther culture in *Datura innoxia*, marking the beginning of in vitro haploid production (Sharp *et.al.,* 1984).

Key historical developments include:

* **1964** – First successful androgenesis reported via anther culture.
* **1970s–1980s** – Application expanded to cereals like barley and rice through cold shock pretreatment and refined media protocols.
* **1990s** – Wide hybridisation methods were developed for wheat, such as wheat × maize crosses, allowing haploid induction via genome elimination.
* **2000s onward** – Molecular and gene-editing-based haploid induction methods discovered, including *MTL* and *CENH3* approaches in maize and Arabidopsis.

These advances transitioned DH technology from experimental systems into mainstream crop breeding pipelines, especially in maize and wheat, where commercial DH production facilities are now widely established.

***D. Importance of DHs in plant breeding and genetics research***

DH lines represent a vital breeding tool due to their complete genetic uniformity, allowing precise phenotypic evaluations and trait mapping (Yan *et.al.,* 2017). In hybrid breeding, DH lines serve as pure parental lines, enhancing hybrid vigour by ensuring maximum heterosis.

The use of DHs in quantitative trait locus (QTL) mapping has been widely documented. For instance, DH populations have facilitated the identification of major QTLs for drought tolerance in wheat and resistance to downy mildew in maize.

In genomic selection, DH lines enable rapid cycle breeding by serving as both training and selection populations, improving accuracy and reducing generation time. The generation of DH lines has also been instrumental in building high-density genetic maps and validating molecular markers for marker-assisted selection (MAS) programs.

Research applications extend to mutagenesis and reverse genetics, where uniform DH backgrounds reduce segregation noise and facilitate mutant identification. This utility has been demonstrated in model crops like barley and maize using TILLING and CRISPR platforms.

**III. Methods of Haploid and Doubled Haploid Production**

***A. In vivo techniques***

***1. Wide hybridisation (e.g., wheat × maize system)***

Wide hybridisation exploits interspecific crosses where one genome is selectively eliminated during early embryogenesis, resulting in haploid progeny from the retained genome. A widely utilised example is the **wheat × maize system**, where maize pollen is used to pollinate wheat ovules. Following fertilisation, maize chromosomes are progressively eliminated during the initial mitotic divisions, producing haploid wheat embryos.

This technique is effective in bread wheat (*Triticum aestivum*), delivering haploid embryo formation rates of 20–40% depending on genotype and environmental conditions (Patial *et.al.,* 2019). Embryo rescue is typically required after pollination, as the hybrid embryo fails to develop into mature seed due to lack of endosperm. Hormone supplementation with 2,4-Dichlorophenoxyacetic acid (2,4-D) has been shown to enhance embryo retention and growth.

***2. Use of haploid inducers (e.g., in maize, rice)***

Haploid inducer lines have revolutionised DH technology, particularly in crops such as maize (Maqbool *et.al.,* 2020). In these systems, specific inducer genotypes, when crossed as male parents, trigger genome elimination in the fertilised egg, resulting in maternal haploids. In maize, the origin of this approach traces back to Stock 6, which showed a natural haploid induction rate of ~1–3%. Through recurrent selection and marker-assisted breeding, modern inducers such as RWS, PHI, and CIM2G027 exhibit haploid induction rates exceeding 10–15%.

In rice, the identification and mutation of *OsDMP* and *OsMTL* have initiated successful haploid induction strategies using genome-editing tools. CRISPR-based knockout of *OsMTL* in male gametes leads to uniparental genome retention and haploid formation at rates of ~6%. Such strategies have opened new avenues for cereals and are being extended to dicots, including tomato and rapeseed.

***3. Genetic engineering approaches***

Genetic manipulation of key regulators of centromere function or fertilisation pathways has proven effective in inducing haploidy. Disruption of *CENH3* through gene replacement in Arabidopsis has induced haploidy by uniparental chromosome elimination due to centromere incompatibility (Ravi *et.al.,* 2010). This method is particularly notable for being genotype-independent and potentially applicable across diverse plant species.

Other genetic strategies include modification of *DMP* (Domain of Unknown Function 679 Membrane Protein) and *MTL*, both of which have demonstrated high utility in maize and are being explored in wheat and rice for cross-species applications. These gene-editing approaches allow targeted development of inducers without conventional breeding cycles, accelerating the adoption of DH technology in non-model species.

***B. In vitro techniques***

***1. Anther culture***

Anther culture involves the direct culture of intact anthers containing microspores at the appropriate developmental stage, typically the uninucleate stage. Upon exposure to stress treatments such as cold, nutrient starvation, or osmotic stress, microspores are reprogrammed to follow a sporophytic embryogenesis pathway.

This method has shown high efficiency in rice, where callus induction frequencies can reach 60–80% depending on the genotype and culture medium. Similarly, in barley and tobacco, anther culture has become a routine DH production method in breeding programs.

***2. Microspore culture***

Microspore culture offers a more refined and direct technique compared to anther culture. Isolated microspores are cultured in a defined medium, and embryogenesis is induced under specific temperature or chemical stress regimes (Touraev *et.al.,* 1997). The technique is particularly well-developed in *Brassica napus* (canola), with embryoid formation rates reaching up to 80% in elite cultivars.

Success has also been documented in crops like barley and wheat, though genotype responsiveness remains a challenge. Pre-treatments such as mannitol stress or heat shock are critical to break the gametophytic developmental pathway and trigger totipotency.

***3. Ovule and ovary culture***

Ovule and ovary culture are used primarily for inducing haploids from female gametophytes via gynogenesis (Keller *et.al.,* 1996). This method is especially effective in species where androgenesis is recalcitrant, such as onion, sugar beet, and cucumber.

In sugar beet, ovule culture has resulted in haploid induction frequencies of up to 25%, with significant improvements achieved through genotype selection and cold pre-treatment. Despite lower overall efficiency than androgenesis, gynogenesis remains valuable for crops with low microspore viability or high floral abortion.

***C. Chromosome doubling methods***

***1. Spontaneous doubling***

Spontaneous chromosome doubling occurs naturally in some haploid plants during mitotic or meiotic divisions. The frequency of spontaneous doubling varies widely among species and genotypes, with barley and tobacco showing relatively high rates (30–70%) while wheat often requires external intervention.

This natural doubling is advantageous as it avoids chemical use, reducing genotoxic risks. However, reliance solely on spontaneous doubling can lead to unpredictable outcomes and variable fertility restoration.

***2. Chemical induction (e.g., colchicine, oryzalin)***

Chemical agents such as colchicine and oryzalin are commonly used to induce chromosome doubling (Hansen *et.al.,* 1996). Colchicine disrupts microtubule polymerisation, arresting cell division at metaphase and promoting endoreduplication. Concentrations between 0.05% and 0.5% colchicine for 4–48 hours are standard across species.

Oryzalin, a dinitroaniline herbicide, has been found to be less toxic and equally effective at inducing polyploidy. In canola and sugar beet, oryzalin has demonstrated doubling efficiencies of up to 90%, with minimal adverse effects on plant development.

***3. Physical methods (temperature shocks)***

Temperature shock, either cold or heat, can facilitate chromosome doubling by affecting spindle fibre formation during mitosis. For example, subjecting microspores to 33–35°C for 24–48 hours enhances embryogenesis in *Brassica napus* and barley. Cold treatments (4°C for 5–7 days) post-fertilisation have also increased spontaneous doubling in cereals such as rice and wheat.

Although less precise than chemical methods, temperature treatments offer a cost-effective and non-toxic alternative, especially in high-throughput systems (Stossi *et.al.,* 2023).

***D. Comparative efficiency and limitations of different methods***

Each DH production method presents unique advantages and challenges. In vivo haploid induction, particularly through maize inducers, offers scalability, high throughput, and is widely adopted in commercial breeding. The method is relatively genotype-independent but limited by the need for specific inducer lines and cross-pollination systems.

In vitro methods provide direct access to gametophytic cells and are ideal for controlled experimentation (Saitou *et.al.,* 2021). Their main limitation lies in strong genotype dependency, especially in cereals like wheat and maize. Culture protocols require optimization of media, stress treatments, and developmental stage synchronization, which may not be feasible for all breeding programs.

Chromosome-doubling methods, while effective, require precise dosing and application to avoid chimerism or sterility. Chemical agents like colchicine, although widely used, are toxic and may pose environmental and health risks. The integration of gene-editing-based inducers and non-toxic doubling strategies could resolve these concerns and enhance overall efficiency.

**IV. Genetic and Molecular Mechanisms Underlying Haploid Induction**

***A. Key genes and loci involved (e.g., MTL, DMP, CENH3)***

The discovery of specific genes responsible for haploid induction has significantly advanced the development of efficient DH systems, especially in major crops such as maize and rice. The central genes identified include *MTL* (MATRILINEAL), *DMP* (DOMAIN OF UNKNOWN FUNCTION 679 MEMBRANE PROTEIN), and *CENH3* (CENTROMERIC HISTONE H3), each functioning through unique biological pathways to trigger haploidy.

***MTL (MATRILINEAL)***

The *MTL* gene encodes a sperm cell-specific phospholipase A1 and plays a crucial role in in vivo haploid induction in maize (Pan *et.al.,* 2024). Loss-of-function mutations in *MTL* disrupt male gamete contribution during fertilisation, leading to genome elimination and the formation of maternal haploids. *MTL* mutants induce haploids at rates ranging from 6% to 15%. This gene is tightly linked to the *qhir1* locus on chromosome 1, which is a major QTL for haploid induction capacity in maize.

***DMP (DOMAIN OF UNKNOWN FUNCTION 679 MEMBRANE PROTEIN)***

*DMP* genes have been identified as another key player in haploid induction. Knockout of *ZmDMP* in maize and orthologous *DMP* genes in dicot crops such as tomato, tobacco, and rapeseed results in the successful induction of maternal haploids. Haploid induction rates of up to 7% in maize using CRISPR-Cas9-mediated *ZmDMP* mutants. These results highlight the conserved nature of DMP-mediated pathways across species.

***CENH3 (CENTROMERIC HISTONE H3)***

The *CENH3* gene encodes a centromere-specific variant of histone H3 and plays an essential role in chromosomal segregation (Maheshwari *et.al.,* 2015). In Arabidopsis, altering the N-terminal tail of *CENH3* or replacing the native gene with a modified version results in centromere dysfunction. This leads to the preferential elimination of chromosomes contributed by the modified centromere parent, producing haploid progeny with high efficiency.

***B. Mechanisms of genome elimination and parthenogenesis***

***Genome elimination***

Genome elimination is a predominant mechanism in both interspecific hybridisation (e.g., wheat × maize crosses) and in vivo haploid induction systems using genetic mutants. During fertilisation, abnormal spindle formation, asynchronous chromatin condensation, or defective centromere recognition can result in the selective loss of one parental genome.

In maize, *MTL* and *DMP* mutations interfere with the paternal genome’s stable integration into the zygote (Evans *et.al.,* 2001). This results in the retention and development of the maternal genome, forming haploid embryos. Similar mechanisms are observed in wide hybridisations, where parental incompatibility causes uniparental chromosome elimination during early embryo development.

***Parthenogenesis***

Parthenogenesis refers to the development of an embryo from an unfertilized egg cell. Although less common in crop species, this pathway is well documented in apomictic plants such as *Hieracium* and *Pennisetum*. Molecular triggers of parthenogenesis include overexpression of *BBM (BABY BOOM)*, a transcription factor that initiates embryogenesis in the absence of fertilisation. Ectopic expression of *OsBBM1* in rice egg cells induced parthenogenetic embryo development.

Although parthenogenesis has limited direct use in major crops at present, it presents a valuable mechanism for the development of clonal seed-based propagation systems and maternal haploid production.

***C. Role of epigenetics and centromere function***

***Epigenetic modifications***

Epigenetic reprogramming plays a vital role during haploid induction, particularly in terms of chromatin remodelling and gene expression regulation during early embryogenesis (Chen *et.al.,* 2022). Hypomethylation of DNA, histone acetylation, and altered small RNA profiles have been observed during the transition from gametophytic to sporophytic development in microspores.

These modifications contribute to the reactivation of embryogenic programs, especially under stress conditions used during androgenesis or microspore culture. For instance, heat and starvation treatments induce oxidative stress that modifies histone marks, promoting embryogenic competence.

***Centromere functionality***

Centromere strength and identity are critical in chromosome retention during zygotic division. In the *CENH3*-mediated haploid induction system, functional asymmetry between parental centromeres causes segregation failure of one genome. The weaker centromere is often lost, resulting in a uniparental haploid progeny.

This centromere-mediated genome elimination has been leveraged to create haploids in Arabidopsis and other model species. Studies on maize and wheat are exploring whether similar centromeric modulation can be adapted for monocots.

***D. Genotype dependency and environmental influence***

Haploid induction efficiency is strongly affected by genetic background. In in vitro methods such as anther and microspore culture, genotype responsiveness varies widely, with some cultivars showing embryoid induction frequencies above 70%, while others fail to respond entirely (Niazian *et.al.,* 2020). In rice and wheat, indica varieties often exhibit lower androgenic response compared to japonica or durum types.

Environmental conditions before and during haploid induction are also significant. Donor plant growth conditions, such as photoperiod, temperature, and nutrient status, influence gamete viability and response. For example, cold pretreatment of spikes or tillers for 3–5 days at 4°C can enhance androgenesis in cereals by 2–3 fold. Similarly, culture environment variables—such as pH, osmotic pressure, and hormone composition—directly affect embryogenesis rates in vitro.

In in vivo systems, maternal genotype significantly influences induction success. Variation in haploid induction rates between 1% and 15% depending on female maize lines used in crosses with the inducer (Prigge *et.al.,* 2011). This suggests the presence of maternal factors that modulate genome retention or elimination following fertilisation.

**V. Application of DH Technology in Crop Improvement**

***A. Accelerating homozygosity and line fixation***

One of the primary advantages of DH technology is the rapid production of completely homozygous lines. Traditional selfing methods require 6–8 generations to achieve near-complete homozygosity, while DH systems accomplish this in a single generation. This reduction in breeding time accelerates the release of new cultivars and supports faster genetic gain.

In maize, homozygous lines developed via DH methods serve as parental inbreds in hybrid breeding programs. DH lines reduce residual heterozygosity and segregating loci, enhancing the genetic purity and uniformity of breeding populations. In wheat, the use of wheat × maize wide crosses has produced stable homozygous lines within 2–3 generations through DH protocols.

***B. Enhancing precision breeding and hybrid development***

DH technology contributes significantly to hybrid development by providing rapid and uniform inbred lines that maximise heterosis (Chaikam *et.al.,* 2019). Since hybrid vigour is dependent on the genetic distance between parental lines, precise and reproducible inbreds are essential for effective hybrid seed production.

DH lines are genetically stable and allow accurate phenotyping under multiple environments. This helps breeders reliably assess combining ability and trait performance, expediting hybrid development cycles. In crops like rice, maize, and canola, hybrid breeding programs now routinely incorporate DH systems for parental line development.

In canola (*Brassica napus*), the use of DH technology has resulted in higher hybrid seed yield, reduced cost per genotype evaluated, and improved hybrid purity. A key commercial advantage is the minimisation of off-types and variability in commercial hybrids.

***C. Use in molecular breeding and marker-assisted selection (MAS)***

DH lines, being completely homozygous, are ideal materials for molecular breeding (Yan *et.al.,* 2017). Marker-assisted selection (MAS) relies on precise genotype–phenotype associations. Segregation in traditional populations can obscure these associations, particularly for complex traits. In contrast, DH populations allow clearer identification of marker–trait linkages due to their genetic uniformity.

Quantitative trait loci (QTL) mapping using DH populations has led to the identification of important loci controlling disease resistance, abiotic stress tolerance, and yield components in major crops. For example, in wheat, DH lines have been used to map QTLs for rust resistance and grain protein content. In maize, MAS has been successfully employed in DH lines to introgress drought and low-nitrogen tolerance traits.

In rice, DH populations derived via anther culture and wide crosses have enabled rapid validation of SSR and SNP markers linked to traits such as blast resistance, submergence tolerance, and grain quality (Pradhan *et.al.,* 2021). These advances increase the reliability of MAS in early-generation selection, significantly reducing breeding cost and time.

***D. Role in genomic selection and genome-wide association studies (GWAS)***

DH technology enhances the effectiveness of genomic selection (GS) and genome-wide association studies (GWAS) by providing high-quality, repeatable phenotypic data. In GS, large populations of DH lines are genotyped and phenotyped, creating robust models that predict breeding values based on genomic profiles.

Using DH lines eliminates variability due to heterozygosity, allowing more accurate heritability estimates. Studies in maize have shown that DH-based training populations improve prediction accuracy in GS models by 10–20% compared to segregating populations.

GWAS relies on precise phenotyping across genetically stable genotypes to detect significant associations between traits and genomic regions. DH populations provide ideal resources for GWAS due to their fixed allelic composition. In barley and wheat, DH panels have been used to map loci for grain yield, heading date, and plant height with high resolution.

***E. Case studies in major crops***

***1. Cereals: maize, wheat, rice***

In maize, DH technology has become an integral part of commercial breeding. The use of inducer lines such as CIM2G027 enables the generation of haploids at rates exceeding 10%, with doubling achieved through colchicine or spontaneous methods (Hooghvorst *et.al.,* 2021). The integration of DH into maize breeding has shortened hybrid development cycles to 3–4 years.

Wheat programs have adopted the wheat × maize system to generate DH lines for biotic stress resistance and quality improvement. This method yields haploid embryos at frequencies ranging from 20% to 40%, depending on genotype and pollination protocol.

In rice, advances in genome editing have enabled in vivo haploid induction using *OsMTL* and *OsDMP* knockouts. These systems deliver haploid induction rates of up to 6% in elite indica lines, offering a rapid route for generating pure lines in both hybrid and varietal breeding.

***2. Oilseeds: canola***

In *Brassica napus*, microspore culture is the preferred DH method due to its high efficiency and scalability (Arabzai *et.al.,* 2025). Protocols allow for embryoid formation rates of 70–90% in responsive genotypes. Canola breeding programs use DH lines extensively for both open-pollinated and hybrid cultivar development. Use of DH systems improves seed uniformity, oil content, and resistance to blackleg and sclerotinia diseases.

DH lines have also contributed to genomic selection efforts, where they enable large-scale genotyping with minimal residual heterozygosity, boosting prediction models’ accuracy.

***3. Vegetables: pepper, cucumber***

In *Capsicum annuum* (pepper), anther culture has been employed to develop DH lines for hybrid seed production and disease resistance breeding. Rates of callus induction and plant regeneration vary widely by genotype, with some cultivars achieving >40% haploid embryo formation. These lines have been used to breed hybrids resistant to *Phytophthora capsici* and tobacco mosaic virus.

In *Cucumis sativus* (cucumber), gynogenesis via ovary and ovule culture has been optimised to generate DH lines in recalcitrant genotypes (Deng et.al., 2020). Regeneration frequencies of 10–20% have been reported, supporting breeding programs targeting traits such as parthenocarpy and powdery mildew resistance.

***4. Emerging uses in legumes and horticultural crops***

Legumes such as chickpea, lentil, and soybean have posed challenges due to low androgenic and gynogenic responses. Despite these hurdles, microspore culture protocols are being optimised. In chickpea, for instance, stress pre-treatments and hormone cocktails have induced embryogenesis in select genotypes.

Horticultural crops like onion, garlic, and eggplant have seen significant progress using gynogenesis-based DH systems. In onion (*Allium cepa*), ovule culture has produced haploids with doubling rates reaching 60% after oryzalin treatment. These DH lines are now being used to develop hybrids with higher pungency and bolting resistance.

**VI. Integration with Modern Breeding Tools**

***A. DH technology and speed breeding***

Speed breeding techniques aim to shorten generation cycles using extended photoperiods, optimised light quality, and controlled temperature regimes (Wanga *et.al.,* 2021). When combined with doubled haploid (DH) technology, this approach dramatically accelerates the delivery of elite lines and hybrids.

Under speed breeding conditions, crops such as wheat and barley can complete a generation in 6–8 weeks. When DH induction is included in this pipeline, homozygous lines can be developed within 3–4 months. This synergy enables breeders to perform multiple breeding cycles per year, significantly increasing genetic gain.

Maize breeding programs using DH and speed breeding protocols have achieved up to threefold increases in selection cycles per unit time compared to conventional methods. In canola, DH lines raised under controlled environments with extended light duration produced seed-ready plants in under 60 days, enabling faster evaluation of hybrid combinations.

Combined pipelines support large-scale selection with minimal field dependency, particularly useful during early stages of cultivar development when speed is crucial (Hu *et.al.,* 2018).

***B. Integration with CRISPR and genome editing tools***

CRISPR-Cas9 and related genome editing platforms have revolutionised precision breeding by enabling site-specific DNA modifications. When integrated with DH technology, these tools facilitate rapid fixation of edited alleles, circumventing the need for prolonged backcrossing and segregation.

In maize, genome-edited haploids have been produced by crossing elite lines with *mtl* or *dmp* knockout haploid inducers, where the paternal genome is eliminated while maternal edits are preserved. Successful transmission of CRISPR-induced mutations in *ARGOS8* and *ZmCCT* genes using this strategy. Editing-induced phenotypes were fixed in the DH stage within a single generation.

In rice, *OsDMP* and *OsMTL*-based haploid induction platforms have been used to fix targeted edits for grain quality and plant height genes. The use of haploid inducers carrying CRISPR machinery allows multiplex editing and efficient trait stacking.

This integration ensures that modified alleles are directly transferred into breeding lines, avoiding transgene segregation and reducing regulatory hurdles associated with genetically modified organisms (Rizwan *et.al.,* 2019).

***C. Use in reverse genetics and functional genomics***

Reverse genetics approaches involve disrupting or modifying genes to determine their function. DH lines provide a uniform genetic background that facilitates phenotype–genotype correlation with high precision.

In functional genomics, DH populations are used to validate candidate genes identified through transcriptomics, proteomics, or GWAS. Their homozygosity reduces the background noise caused by segregation, enabling clearer interpretation of phenotypic effects.

TILLING (Targeting Induced Local Lesions in Genomes) platforms have benefited from the use of DH lines. In barley, mutation libraries created via EMS mutagenesis were stabilised through DH production, allowing high-throughput screening for traits like disease resistance and chlorophyll content.

In maize, DH-based mutant libraries have supported functional analysis of key agronomic traits, including kernel development and nitrogen-use efficiency. CRISPR-edited DH lines have similarly been employed to dissect gene regulatory networks and validate transcription factors involved in stress responses.

***D. Application in polyploid and recalcitrant species***

Polyploid crops such as wheat (hexaploid), potato (tetraploid), and banana (triploid) pose challenges for classical breeding due to complex inheritance patterns and delayed fixation of traits. DH technology offers a solution by simplifying the genome to a diploid state before recombination or trait stacking.

In hexaploid wheat, DH lines derived via wheat × maize crosses have enabled rapid selection for traits such as high zinc content, drought tolerance, and rust resistance. Chromosome doubling stabilises recombinant genomes and facilitates downstream hybrid development.

Potato, though vegetatively propagated, has been subjected to haploid induction protocols to create diploid lines suitable for true seed production (Sharp *et.al.,* 1984). These diploid lines are then subjected to genome editing and crossed with other elite lines to develop uniform progeny.

Recalcitrant species such as chickpea, onion, and sunflower often respond poorly to in vitro regeneration or have low transformation efficiencies. Integration of DH and genome editing in these crops has been explored using ovule culture or wide hybridisation with genome elimination. In onion, for instance, gynogenesis followed by oryzalin treatment has produced DH lines with high frequency, enabling faster hybrid development and trait introgression.

Application of stress treatments and use of anti-mitotic agents such as colchicine and oryzalin have improved doubling rates in these species, supporting the use of DH platforms even in genetically complex or poorly responding crops (Chaikam *et.al.,* 2020).

**VII. Challenges and Limitations**

***A. Genotype dependency and recalcitrance in some species***

The success of haploid and doubled haploid (DH) technologies often hinges on the genetic background of donor plants. Genotype-specific responses to both in vivo and in vitro haploid induction present a significant challenge, especially in crops with diverse genetic pools.

In anther and microspore culture systems, only a subset of genotypes demonstrate high embryogenic potential. For example, in barley, cultivars such as ‘Igri’ exhibit callus induction rates above 80%, while others fail to produce any viable embryos. In rice, indica genotypes show poor response compared to japonica types, primarily due to differences in microspore viability and stress response pathways.

Similar limitations exist in maize haploid induction. Although haploid inducer lines like RWS and PHI have been broadly successful, maternal genotype influences the efficiency of haploid production and embryo viability. Significant variation in induction frequency (1–15%) across different female lines, suggesting the presence of maternal factors affecting genome elimination.

In crops such as soybean, chickpea, and lentil, high levels of recalcitrance to both androgenesis and gynogenesis limit the widespread use of DH methods (Hale *et.al.,* 2022). Efforts to overcome genotype dependency include pre-treatment optimisation, donor plant conditioning, and development of more universally responsive inducers.

***B. Low efficiency and technical difficulties in in vitro methods***

In vitro haploid production techniques, including anther culture and microspore culture, are technically demanding and often suffer from low reproducibility. Critical steps such as isolation of viable microspores, synchronisation of developmental stages, and optimisation of culture media require precise execution and constant monitoring.

Even in responsive species, the induction of embryogenesis is highly sensitive to external stress factors such as temperature, osmotic pressure, and hormone balance. Minor deviations in culture conditions can lead to callus formation instead of embryoid development or result in necrosis. In wheat, microspore embryogenesis remains inconsistent, with embryoid formation rates below 10% in most elite cultivars (Delporte *et.al.,* 2014).

Contamination, somaclonal variation, and albinism are additional problems encountered during tissue culture. In cereals like rice and barley, the frequency of albino plantlets can exceed 30%, reducing the number of viable DH lines that can be regenerated.

These issues necessitate skilled personnel, sterile conditions, and extensive genotype-specific protocol development, making large-scale implementation of in vitro DH production a major challenge.

***C. Cost, infrastructure, and scalability issues***

Establishing and maintaining DH production facilities involves significant financial and logistical investment (Min *et.al.,* 1999). Infrastructure requirements include controlled growth chambers, sterile culture rooms, embryo rescue labs, and trained technical staff. High-input components such as growth regulators, media supplements, and chromosome-doubling chemicals add to operational costs.

In maize, while in vivo systems are more scalable, they still require maintenance of inducer lines, controlled pollination, embryo rescue, and chromosome doubling protocols. Studies estimate that DH line production can cost $40–$70 per line, depending on efficiency and resource availability. These costs may limit access to small- and medium-scale breeding programs.

The use of expensive chemical agents like colchicine or oryzalin for chromosome doubling further raises safety and disposal concerns. Automating embryo rescue and high-throughput screening may lower costs in the future, but current scalability remains a concern, particularly in less-resourced research settings.

***D. Potential genetic abnormalities and epigenetic changes***

DH production processes, especially those involving stress-induced embryogenesis, can introduce genetic and epigenetic changes (Karami *et.al.,* 2010). Spontaneous or chemically induced chromosome doubling may cause mitotic irregularities, aneuploidy, or segmental duplications.

In wheat, cytological analyses of DH lines have revealed meiotic abnormalities and occasional structural rearrangements, potentially affecting fertility and trait stability. Chemical doubling using colchicine may induce point mutations and chromosomal bridges due to its impact on spindle fibres and chromosome segregation.

Epigenetic reprogramming under stress conditions in anther or microspore culture can result in altered gene expression, DNA methylation, and histone modification. These changes may lead to unpredictable phenotypes, especially under field conditions. Microspore embryogenesis in barley is associated with altered reactive oxygen species (ROS) profiles and caspase-like activity, contributing to cell death or developmental abnormalities.

While many of these changes may be stabilised after a few generations, they complicate trait evaluation and can delay varietal release in some breeding programs.

***E. Regulatory and acceptance issues in transgenic approaches***

Integration of DH systems with transgenic or genome editing platforms raises biosafety and regulatory concerns (Guru *et.al.,* 2023). Although CRISPR-based edits can be transgene-free, public perception and legal classification often conflate them with traditional GMOs, leading to delays in approval and commercialisation.

Haploid inducers modified using CRISPR to carry Cas9 and guide RNAs have shown promise in maize and rice for editing target loci and recovering edited haploids. Despite this, such lines fall under existing GMO legislation in many countries, requiring extensive risk assessments, environmental impact studies, and multi-year trials.

Export markets may impose additional restrictions on the use of gene-edited lines, impacting the economic feasibility of DH-enabled trait delivery. Consumer concerns and variable regulatory frameworks across regions further limit the broad deployment of transgenic DH technology.

Efforts are ongoing to clarify the regulatory status of precision-edited, non-transgenic DH lines, especially those derived using transient expression systems or DNA-free genome editing tools.

**VIII. Recent Advances and Future Directions**

***A. Novel haploid inducer lines and gene editing technologies***

Development of high-efficiency haploid inducer lines, especially in maize and rice, has transformed doubled haploid (DH) technology into a scalable, routine tool for breeders (Delzer *et.al.,* 2024). Novel inducers engineered through targeted mutagenesis of key genes such as *MTL* (MATRILINEAL), *DMP* (DOMAIN OF UNKNOWN FUNCTION 679 MEMBRANE PROTEIN), and *PLD3* (PHOSPHOLIPASE D3) have demonstrated higher induction frequencies and broader applicability across genetic backgrounds.

In maize, inducer lines like CIM2G027 and CAUHOI have reached haploid induction rates of 10–15%. Mutations in *ZmDMP* not only increase induction frequency but also offer maternal haploid recovery without affecting seed viability. These lines are being optimised for use in tropical and subtropical conditions through recurrent selection and backcrossing.

In rice, CRISPR-Cas9-mediated knockout of *OsMTL* and *OsDMP* has led to the development of male haploid inducers with average induction rates of 6%. Efforts to pyramide such mutations and combine them with Cas9 transgenes allow direct genome editing and haploid induction in a single step.

Gene-edited inducers also offer new strategies for non-model crops through cross-species transfer of haploid induction traits. Recent studies have shown the successful adaptation of DMP-based systems in tomato, tobacco, and soybean, indicating broad cross-utility.

***B. Use of machine learning and AI in DH-based breeding pipelines***

Artificial intelligence (AI) and machine learning (ML) algorithms are increasingly applied to optimize DH breeding workflows (Farooq *et.al.,* 2024). These tools assist in identifying genotypes with higher haploid induction responsiveness, predicting embryo viability, and improving selection accuracy.

Deep learning models trained on image datasets are used to automate embryo detection and classification during haploid screening in maize and rice. Convolutional neural networks (CNNs) have achieved embryo classification accuracies above 95%, significantly reducing manual labour and increasing throughput.

ML algorithms, including support vector machines and random forest classifiers, are also employed to analyse large-scale phenotypic and genotypic datasets from DH populations. These models predict trait performance under variable environmental conditions, facilitating the selection of elite lines during early breeding stages.

Integration of AI into DH platforms is accelerating genotype-to-phenotype predictions, reducing time and cost associated with traditional trial-and-error-based breeding.

***C. Synthetic biology approaches to enhance haploid induction***

Synthetic biology tools are being developed to precisely control fertilisation and embryogenesis, offering new frontiers in haploid induction (Shen *et.al.,* 2023). Synthetic promoters and modular gene circuits allow spatial and temporal control of key haploid induction genes, minimising off-target effects.

Designer centromeres, created through synthetic engineering of centromeric histone complexes like *CENH3*, have been used to trigger selective genome elimination in Arabidopsis. Reconstitution of these systems in crops like wheat and maize is underway, aiming to establish synthetic haploid induction without the need for traditional hybridisation.

Gene circuits regulating the expression of embryogenesis-promoting genes such as *BBM* (BABY BOOM) and *WUS* (WUSCHEL) are being explored to induce parthenogenesis or haploid development in recalcitrant crops. Transient expression of these genes using synthetic modules can initiate embryo formation without stable transformation, thus bypassing regulatory barriers associated with GMOs.

This modularity of synthetic biology platforms enables programmable haploid induction, particularly valuable in species with poor natural induction frequencies.

***D. Potential in climate-resilient and orphan crop breeding***

As climate change accelerates, demand for breeding climate-resilient and underutilised (orphan) crops is rising (Singh *et.al.,* 2024). DH technology offers a rapid route to fix stress-tolerant traits and broaden genetic diversity in crops lacking commercial breeding support.

Millets, sorghum, and pulses such as cowpea and mung bean have shown limited responsiveness to traditional breeding. DH systems using wide hybridisation, gynogenesis, and genome editing are being tested to overcome reproductive barriers. In sorghum, early attempts at gynogenesis and anther culture have achieved haploid regeneration in select genotypes with optimised pre-treatments.

Orphan crops such as teff (*Eragrostis tef*) and amaranth (*Amaranthus spp.*) are being targeted for DH line development through microspore culture and emerging in vivo systems. By integrating DH methods with speed breeding and genomic selection, these crops can be improved for drought resilience, early maturity, and nutritional enhancement.

Establishing DH platforms in these species ensures their inclusion in modern agriculture systems, promoting food security in marginal environments.

***E. Future prospects for automation and high-throughput DH production***

Automation technologies are transforming DH workflows by increasing efficiency, scalability, and reproducibility (Rachakatla *et.al.,* 2022). Robotic platforms are being deployed for tasks such as pollination, embryo rescue, media preparation, and chromosome doubling.

High-throughput embryo sorting using flow cytometry and image-based recognition allows rapid identification and transfer of viable haploids (Dermail *et.al.,* 2024). Fluorescent markers, such as GFP or RFP, expressed in embryo sacs or endosperm, enable non-destructive sorting of haploid and diploid seeds in maize and wheat.

Liquid handling robots and microfluidic culture systems are used in microspore culture pipelines for canola and barley, reducing human error and increasing consistency. Automated chromosome doubling chambers with controlled exposure to colchicine or oryzalin improve treatment precision and reduce chemical waste.

Future advancements in biofoundry-style breeding platforms are expected to integrate genomics, robotics, and AI to create fully automated DH production systems. These developments promise faster cultivar turnover, reduced breeding costs, and expanded accessibility of DH technology.

**IX. Conclusions**

Doubled haploid (DH) technology has emerged as a transformative tool in modern crop improvement, enabling rapid development of completely homozygous lines and accelerating breeding cycles. Through both in vivo and in vitro systems, DH approaches facilitate precise trait fixation, enhance hybrid breeding, and support molecular applications such as marker-assisted selection, genomic selection, and genome editing. Recent advancements in gene-based haploid inducers, integration with CRISPR-Cas systems, and automation technologies have further expanded the efficiency and applicability of DH systems across diverse crop species, including cereals, oilseeds, and vegetables. Despite challenges such as genotype dependency, low efficiency in recalcitrant species, and regulatory concerns in transgenic systems, continued innovation in synthetic biology, AI-driven phenotyping, and speed breeding platforms offers promising avenues for overcoming existing limitations. As global food demand rises, DH technology remains pivotal for delivering resilient, high-yielding cultivars with enhanced genetic gains under changing climatic conditions.

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Details of the AI usage are given below:

1.

2.

3.

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