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Review Article

Doubled Haploid Technology for Genetic Improvement in Barley (*Hordeum vulgare*): Anther Culture and Beyond

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Abstract

Anther culture is an important technology in plant breeding which leads to the production of haploid and doubled haploid plants. The introduction of many cultivars in barley (*Hordeum vulgare*) has been made possible by this technology. Genetic improvement in barley has been greatly aided by doubled haploid (DH) technology, which has made it easier to create linkage maps, perform genetic analysis, and map QTLs. Low embryogenic potential and high albino plantlet frequency are two issues that still limit the effectiveness of DH generation in some genotypes, even with several improvements made to anther culture techniques. To overcome these constraints and ensure the broad application of DH breeding in barley, more optimization is required to improve the viability of green plantlets. Key research needs will be addressed by this review, including different work done by researchers to decrease albinism, increase the effectiveness of haploid induction, and incorporate molecular techniques into DH technology. This review aims to increase DH output, broaden its use in breeding programs, and provide a more efficient framework for applying DH technology in barley research by methodically examining existing methods and suggesting creative solutions.

Key words: Androgenesis, CRISPR/Cas9, doubled haploid, genomics, *Hordeum vulgare*

1. Introduction

Barley (*Hordeum vulgare*), a cereal crop of profound agronomic and economic significance, is cultivated across diverse edaphoclimatic spectra, exhibiting remarkable adaptability to semi-arid, temperate, and saline-prone environments (Ghazy *et al*., 2024). Characterized by its intrinsic resilience to drought, osmotic stress, and extreme temperature fluctuations, barley holds immense potential in climate-resilient agriculture, serving as both a staple food source and a primary substrate in malting, brewing, and livestock (Bishnoi *et al*., 2022). Its innate plasticity in morphological and physiological responses to abiotic stress underscores its pivotal role in sustainable cropping paradigms, particularly within regions affected by erratic rains and escalating soil salinization (Ali *et al*., 2022). Barley is a versatile cereal crop with extensive applications in food, beverage, and industrial sectors, making it integral to global agriculture (Lukinac and Jukić, 2022). Widely used in the brewing industry, barley serves as a primary ingredient for malt production in beer and whiskey, owing to its high enzymatic activity and starch composition. In addition to its role in livestock feed, where it provides essential energy and protein for animal nutrition, barley is gaining prominence in functional foods due to its rich nutritional profile (Geng *et al*., 2022).

As a whole grain, it is an excellent source of dietary fiber, β-glucans, antioxidants, and essential vitamins, contributing to cardiovascular health, improved digestion, and glycemic regulation (Patial *et al*., 2023c, 2024). Recent studies highlight its potential in gluten-free alternatives, catering to individuals with celiac disease or gluten sensitivity through specific breeding programs aimed at developing low-gluten barley varieties (Tanner *et al*., 2016). Furthermore, barley's industrial applications include its use in biofuel production, biodegradable packaging, and nutraceuticals, reflecting its adaptability beyond traditional food systems. As a climate-resilient crop, barley thrives in marginal lands, requiring minimal inputs while enhancing soil structure and nitrogen cycling, making it an essential component of sustainable agricultural frameworks (Patial *et al*. 2018). Its ability to withstand drought, salinity, and extreme temperatures underscores its relevance in future food security strategies, ensuring stable yields in unpredictable climatic conditions (Bishnoi *et al*., 2022).

Haploids are plants with a gametophytic chromosome number and doubled haploids are haploids that have undergone chromosome duplication (Kiełkowska *et al*., 2018). Haploids occur spontaneously at a low frequency, or they can be induced by several methods, such as *in-vivo* modified pollination methods (wide hybridization, chromosome elimination, pollination with irradiated pollen, etc.) and by *in-vitro* culture of immature gametophytes (Gandhi *et al*., 2023; Patial *et al*., 2022; Patial and Verma, 2024). The production of haploids and DHs through gametic embryogenesis allows a single-step development of complete homozygous lines from heterozygous parents, shortening the time required to produce homozygous plants in comparison with the conventional breeding methods that employ several generations of selfing (Germanà, 2011). The pure line is mandatory for developing a hybrid variety and developing a pure line requires several generations by conventional approach (Ayiecho and Nyabundi, 2025). DH technology cut the duration for the homozygous pure line developed within a single generation (Datta, 2005; Gandhi *et al*., 2023; Patial and Verma, 2024).

Doubled haploid technology has improved and accelerated the breeding of new varieties and hybrids. The technique is being used in different crop species such as barley, rapeseed, maize etc (Jiang *et al*., 2025; Patial and Pal, 2017; Weyen, 2009). Microspore culture and broad hybridization techniques are frequently used in wheat (*Triticum aestivum*) (Patial *et al*., 2017, 2016) to create DH lines with improved yield potential and disease resistance. DH technology is essential in hybrid breeding programs because maize (*Zea mays*) mostly depends on *in-vivo* haploid induction followed by colchicine treatment to restore fertility. While Brassica species (such as *B. napus* and *B. oleracea*) make considerable use of microspore culture in oilseed and vegetable breeding, anther culture and isolated microspore culture have been optimized for quick homozygous line creation in rice (*Oryza sativa*) (Arabzai *et al*., 2025). Similar to this, anther culture and wide hybridization techniques help triticale (*Triticosecale* Wittm.) improve genetically more quickly (Kruppa *et al*., 2023).

Anther culture (androgenesis) is an important method for DH production in many crops because of its simplicity thereby allowing large-scale anther culture establishment and application to a wide range of genotypes . Under the appropriate conditions, haploid microspores undergo developmental transition to form DH plants. Instead of developed pollen grains, an embryo or callus is formed as a result of the consecutive divisions. These embryos often produce either spontaneous DHs or with cholchicine treatment DH plants with double chromosome set are produced. A number of critical steps are involved in anther culture, such as callus induction, plant regeneration, donor plant selection, anther pre-treatment and chromosomal doubling. Researchers are investigating methods including altering growth regulators, optimizing stress treatments, and incorporating molecular tools like CRISPR/Cas9 to increase regeneration success rates in order to improve the efficiency of barley androgenesis (Patial *et al*., 2023b).

Barley reacts favorably to a number of DH techniques, such as anther culture, microspore culture, and wide hybridization (*Hordeum bulbosum* method). Among these, anther culture is one of the most widely used methods since it effectively induces androgenesis and can yields more DH plants (Islam *et al*., 2023). However, albino plantlet production is still a major obstacle, and it calls for stress management and medium composition adjustment.

For different biotechnological programs like chromosome mapping, QTL mapping, marker-assisted selection, marker-assisted backcrossing, mutation breeding,

genome-wide association study, genomic engineering, and genome editing, androgenesis based haploid and double haploid plants are being used (Islam *et al*., 2023). Marker-assisted breeding along with the DH technique has been successfully utilized in the various breeding programs to cut short the breeding cycle (Patial *et al*., 2023a; Wessels and Botes, 2014). The homozygous condition, which permits transgene or mutation stabilization in the genome, is reached in a significantly shorter time (2-3 years) by the use of DH breeding than by traditional methods. In conventional breeding, recessive mutations often remain obscured within heterozygous individuals, as their phenotypic effects do not manifest until successive generations undergo segregation. This delay in expression complicates trait selection and necessitates extensive breeding cycles to isolate desirable recessive alleles effectively (Lamichhane and Thapa, 2022). However, DH plants, characterized by their complete homozygosity, circumvent this issue by carrying two identical copies of each allele, ensuring that recessive mutations express immediately upon plant development. This immediate phenotypic manifestation allows breeders to rapidly identify loss-of-function mutations and assess their impact on agronomic traits without the need for prolonged generational screening (Alam *et al*., 2024) . The streamlined nature of DH technology significantly expedites the breeding process, providing an efficient platform for selecting beneficial genetic variations while eliminating undesirable traits early in cultivar development. Furthermore, the fixation of recombination events within DH lines enhances the accuracy of quantitative trait locus (QTL) mapping and genomic selection (GS) strategies, offering a robust foundation for molecular breeding programs aimed at improving stress tolerance, disease resistance, and yield potential in different crops (Trampe *et al*., 2022). By integrating DH plants into breeding frameworks, researchers can optimize genetic improvement, reducing time and resources required for stabilizing elite lines and accelerating the deployment of enhanced cultivars in commercial agriculture (Kumar *et al*., 2020; Patial *et al*., 2019, 2023b).

By enabling the quick creation of homozygous lines in a single generation, DH technology has become a fundamental component of contemporary barley breeding, greatly boosting genetic progress (Patial *et al.,* 2015; Patial *et al.,* 2017). DH technology speeds up the selection of top performing cultivars with better agronomic features in contrast to traditional breeding methods that take several generations to attain genetic fixation (Patial and Pal, 2017). Through the optimization of haploid induction rates, green plantlet regeneration, and genomic stability, the combination of anther culture procedures and molecular tools, such as CRISPR/Cas9 gene editing, has significantly improved the efficiency of DH production (Impens *et al*., 2023). Nevertheless, significant obstacles like low embryogenic potential, genotype dependence, and high frequency of albino plantlets still prevent DH technique from being widely used in barley breeding programs. To fully utilize DH technology in climate-resilient and sustainable agriculture, these constraints must be addressed by multiplex gene editing, predictive breeding models, and protocol improvement. This paper highlights the future approaches for integrating molecular technologies to better DH applications in barley improvement, critically analyzes the developments in DH technique, and investigates the function of GS and marker-assisted selection (MAS) in improving breeding efficiency.

2. Doubled Haploid Technology in Barley vs. Other Crops: A Comparative assessment

Barley primarily uses androgenesis, which uses anther or microspore culture to produce haploid plantlets, whereas wheat and maize primarily rely on wide hybridization, where haploidy is induced through interspecific crosses (e.g., maize × wheat) (Devaux, 2021). The efficacy of haploid induction varies greatly between crops, with barley demonstrating genotype-dependent success rates in anther culture, making the procedure very variable and needing adjustment based on specific cultivars (Han *et al*., 2020b). On the other hand, *in- vivo* haploid induction techniques, especially haploid inducer lines like Stock 6, are advantageous for maize since they guarantee more dependable and effective haploid production (Liu *et al*., 2022). The prevalence of albino plantlets, which drastically lowers green plant regeneration and overall DH efficiency, is a serious obstacle in barley's DH production. In contrast, wheat exhibits a reduced incidence of these problems. These discrepancies highlight the species-specific adaptations in doubled haploid technology and its relevance in modern genetic enhancement tactics.

Table 1 details comparative analysis of DH production across barley, wheat, maize, and rice, highlighting key breeding applications, preferred DH techniques, and major challenges.

Table 1: Comparison of doubled haploid technology in major crops

Crop	Breeding applications	Preferred doubled techniques	Efficiency of haploid induction	Albino plantlet formation	Chromosome doubling	References
Barley (*Hordeum vulgare*)	Climate-resilient cultivars, malting quality improvement	Predominantly anther/microspore culture	Genotype-dependent success; moderate regeneration	High occurrence, reducing DH production efficiency	Colchicine or spontaneous doubling	Han *et al.* (2020b)
Wheat (*Triticum* spp.)	Rust resistance, stress tolerance, high yield breeding	Wide hybridization (wheat × maize) and anther culture	High efficiency in wheat × maize crosses	Occasional albino plantlets, but lower than barley	Colchicine or chemical induction	Guan *et al.* (2024)
Maize (*Zea mays*)	Hybrid vigor improvement, drought tolerance	*In-vivo* haploid induction using Stock 6 genotype	Highly efficient with Stock 6 haploid inducer	Rare albino plantlets	Spontaneous doubling is more frequent	Zhang *et al.* (2008)
Rice (*Oryza sativa*)	Blast resistance, submergence tolerance	Anther/microspore culture	Moderate efficiency with anther culture	Occasional albino plantlets	Colchicine or spontaneous doubling	Lantos *et al.* (2023)

3. Leading Research Labs Advancing Haploid Development in Barley

The term "androgenesis" was introduced by the German physiologist Max Verworn (Verworn, 1891) which is used to describe the production of haploids through anther culture. The haploids and DHs produced through anther culture served many purposes of basic and applied research. Globally, DH technology has gained widespread adoption with efforts to improve genetic traits in a variety of crops, especially cereals like rice, wheat, maize, and barley (Ren *et al.*, 2017). Significant progress has been made in DH breeding by nations like the United States, Canada, Germany, France, India, China, Australia, and Mexico, each of which has concentrated on crop-specific applications tailored to regional agricultural needs (Ordon and Friedt, 2019; Patial *et al.*, 2023a; Samantaray *et al.*, 2021). High-yield hybrid cultivars have been developed in North America as a result of research into maize DH technology, especially in the US and Canada, while breeding efficiency has been maximized in wheat and barley DH programs. Improved maize hybrids with DH lines as parental lines have been released in Africa (Chaikam *et al.*, 2018). Europe has advanced haploid induction techniques and led the way in DH research in barley, rapeseed, and vegetable crops, especially in Germany and France (Kasha and Maluszynski, 2003). China and India have made significant investments in DH rice and wheat breeding throughout Asia (Samantaray *et al.*, 2021), enhancing genetic diversity and stress tolerance characteristics. Australia has used DH technology to create drought-resistant wheat and barley cultivars (Weyen, 2009) because of its arid climate. Refining DH techniques, improving haploid induction efficiency, and incorporating molecular breeding tools like Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9 (CRISPR-Cas9) and MAS for genetic improvement have all been made possible by top research institutions like International Maize and Wheat Improvement Center (CIMMYT, Mexico), International Center for Agricultural Research in the Dry Areas (ICARDA, Lebanon, Syria), Indian Agricultural Research Institute (India), Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (India), John Innes Centre (United Kingdom), and Chinese Academy of Agricultural Sciences (China). These initiatives highlight how important DH technology is on a global scale for boosting crop development initiatives.

Haploid development in barley is being actively studied by a number of research labs and universities throughout the world, with an emphasis on DH technology, anther culture, and microspore culture to improve breeding efficiency. Among the top labs are the following:

- Saaten-Union Resistenzlabor GmbH (Germany) - Specializes in DH breeding for barley and wheat, integrating molecular marker technologies (Weyen, 2009).
- The Leibniz Institute of Plant Genetics and Crop Plant Research (Germany) - Work in microspore culture and androgenesis. This institute explores novel methods to overcome albino plantlet formation and optimize green plant regeneration in barley DH protocols (Ahmadli *et al*., 2023).
- The John Innes Centre (England) - Conducts advanced research on haploid induction and genomic selection in barley breeding (Bozorgipour and Snape, 1992).
- International Maize and Wheat Improvement Center (CIMMYT, Mexico) - Works on DH methodologies for cereal crops, including barley (Li *et al*., 2013).
- Chinese Academy of Agricultural Sciences (China)- Engages in haploid induction research, integrating CRISPR/Cas9 for DH barley improvement (Tang *et al*., 2023).
- The University of Adelaide (Australia) - Develops DH lines for drought and disease resistance using advanced breeding techniques (Eliby *et al*., 2022).
- Institute of Plant Genetics, Polish Academy of Sciences (Poland) - Focuses on anther culture and microspore embryogenesis for DH barley production (Bocianowski *et al*., 2003).
- The Crop Research Institute (Prague-Ruzyne, Czech Republic) - Actively involved in anther culture studies, this institute works on optimizing stress treatments and media composition to improve embryo development and plant regeneration efficiency in barley (Ohnoutková and Vlčko, 2020).
- Indian Council of Agricultural Research-Indian Agricultural Research Institute (Regional Station, Shimla), and Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (India) - DH development in wheat using *Imperata cylindrica* mediated doubled haploidy breeding (Patial *et al*., 2023b).

4. Comparison of *in-vitro* and *in-vivo* Doubled Haploid Technologies in Barley

In-vitro and *in-vivo* DH technologies differ in their approach to haploid induction, chromosome doubling, and efficiency in breeding programs (Table 2). *In-vitro* DH technology includes inducing haploid embryos in a controlled laboratory setting, mostly through anther and microspore culture. This method involves the embryogenic transformation of stressed microspores into haploid plantlets, which are then doubled by means of spontaneous chromosome doubling or colchicine. However, issues including low embryogenic potential, high incidence of albino plantlets, and genotypic specificity call for constant improvement in environmental conditioning, hormone management, and medium composition (Cistué *et al*., 1998). Conversely, *in-vivo* DH technology, widely adopted in cross-pollinated crops like maize, is increasingly being explored through the use of haploid inducer lines (Zhang *et al*., 2008). Unlike *in-vitro* methodologies, which necessitate anther or microspore culture under controlled conditions, *in-vivo* haploid induction exploits chromosome elimination mechanisms, often mediated through haploid inducer lines or distant hybridization strategies. This approach ensures genetic fidelity by mitigating somaclonal variation, a frequent complication in tissue culture-based techniques, thereby preserving the intrinsic genomic integrity of elite barley cultivars (Ishii *et al*., 2016). Additionally, haploid inducer-based pollination, often incorporating wide hybridization methodologies with species such as maize, facilitates haploid embryo formation, allowing for spontaneous or chemically induced chromosome doubling. An alternate strategy for producing DH in barley is irradiated pollen-induced parthenogenesis (PDF) Irradiated Pollen-Induced Parthenogenesis for Doubled Haploid production in sunflowers (*Helianthus* spp.). This method is especially helpful when traditional androgenesis techniques like anther culture and microspore culture are limited by genotype dependency or poor regeneration efficiency. By encouraging unfertilized egg cells to develop into embryos without the pollen donor's genetic input, this method takes advantage of haploid embryo development. Improved breeding efficiency across a range of barley

genetic backgrounds is ensured by *in-vivo* haploid induction, which shows greater success rates and less genotype dependency than *in-vitro* methods.

Table 2: Comparison of *in-vitro* and *in-vivo* doubled haploid technologies [adopted from Seguí-Simarro *et al.* (2021)]

Features	*In-vitro* DH Technology	*In-vivo* DH Technology
Method of Haploid Induction	Uses anther culture, microspore culture, or gynogenesis to induce haploid embryos in controlled environments	Relies on haploid inducer lines that trigger haploid embryo formation through selective pollination
Efficiency	Highly dependent on genotype, media composition, and environmental conditions	More consistent across genotypes
Chromosome Doubling	Chemical agents like colchicine or spontaneous doubling in culture	Natural doubling or induced through chemical treatments post-haploid induction
Challenges	High albino plantlet frequency, low embryogenic potential, and genotype dependency	Requires haploid inducer lines, which may limit genetic diversity
Genetic Stability	Can introduce somaclonal variation due to tissue culture stress	Maintains higher genetic fidelity as haploids develop naturally

5. Historical Overview of Anther Culture Technique in Barley

Bergner observed first sporophytic haploid in 1921 in the weed species of *Datura stramonium* L. which was reported by Blakeslee *et al.* (1922). In 1974, Kasha reported the spontaneous development of haploids in over 100 angiosperm species. The frequency of spontaneous haploids is, however, too low for practical application in breeding. About 40 years after the identification of the first natural haploid, Guha and Maheshwari (Guha and Maheshwari, 1964) discovered the possibility of *in-vitro* culture of immature anthers of the *Solanaceous* species *Datura innoxia* to alter the normal gametophytic development of microspores into a sporophytic one. It has paved the way to extensive research on anther culture which was particularly successfully in the *Solanaceae*, *Brassicaceae* and *Gramineae*. Historically, anther culture has been explored since the 1970s, with advancements in protocols and media formulations enhancing its efficiency. Barley, rapeseed (*Brassica napus* L.), tobacco (*Nicotiana* spp.) and wheat (*Triticum aestivum* L.) are considered to be the model species to study microspore embryogenesis due to their high regeneration efficiency (Forster *et al.*, 2007), however, other species such as *Arabidopsis*, many woody plants or members of legume family, still remain recalcitrant to this type of *in-vitro* morphogenesis (Bajaj, 1990).

Research on barley anther culture has seen contributions from various scientists over the years (Fig. 1). These researchers have significantly advanced the DH technology making barley anther culture a cornerstone in plant breeding and genetic research.

Clapham reported haploid plants from anther culture in barley for the first time in 1973 (Clapgham *et al.*, 1973). Doubled haploidy breeding has produced a number of varieties in Australia, such as the barley varieties Dhow, Sloop SA, Flagship, Navigator, Skipper, and Spartacus. In barley androgenesis studies, the winter barley cultivar "Igri" is frequently used as a model genotype which produces the most green double haploid plants (Li and Devaux, 2001).The research on barley anther culture from 1960 till date has been a journey of innovation and refinement. During the 1960's upto1970's the goal of early research was to determine whether anther culture could produce haploid plants. Techniques for callus formation and haploid plant regeneration in barley were developed and standardized by researchers such as Clapham and Dale. In the 1980's developments in media formulations, including the identification of N6 medium, which increased anther culture's effectiveness,

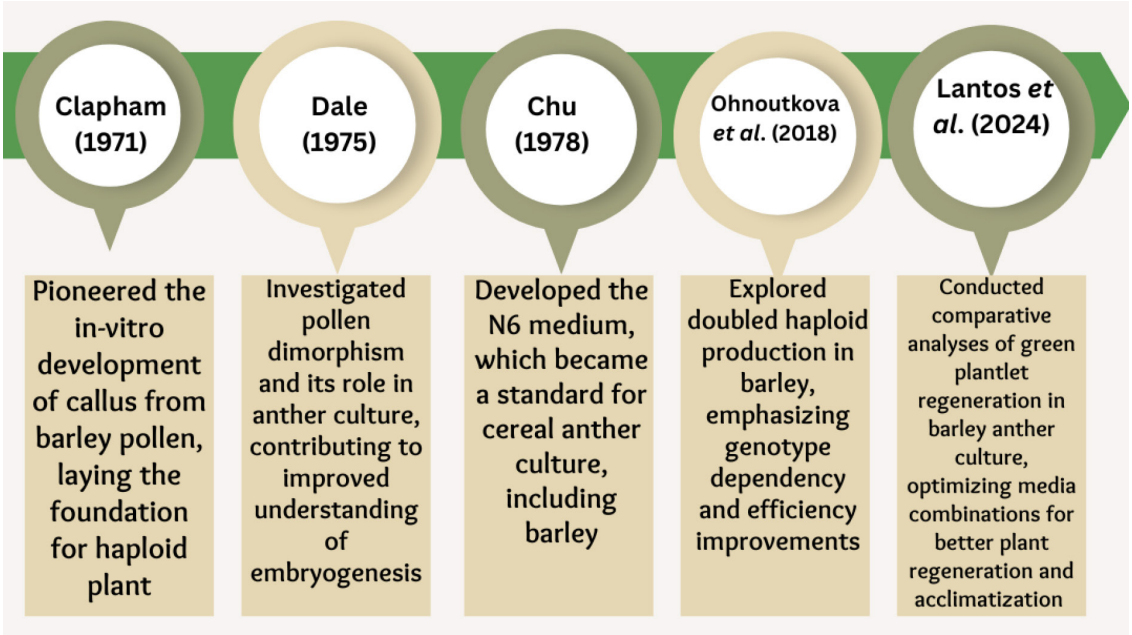

Fig. 1. Contribution of scientists in barley anther culture research

took place. Genotype dependency was investigated, and growth conditions were improved for better results. The 1990's and 2000's saw a change in emphasis toward improving green plantlet regeneration and decreasing the prevalence of albino plantlets. Colchicine-based methods for doubling chromosomes in haploid plants were also improved. The 2010's saw the integration of molecular biology techniques with anther culture studies, which improved our comprehension of genetic stability and androgenesis (Mishra and Rao, 2016). Barley breeding programs began to rely on doubled haploid plants. In 2020s, improving plant acclimation rates and regeneration efficiency has been the focus of research and high-quality doubled haploid plants for breeding and research have been produced.

Androgenesis, which entails cell fate redirection within the microgametophyte, is employed widely for genetic gain in plant breeding programs. Androgenesis-responsive species provide systems for studying cell cycle regulation, meiotic recombination, and apozygotic embryogenesis within plant cells. Past research on androgenesis has focused on protocol development with emphasis on temperature pretreatments of donor plants or floral buds, and tissue culture optimization because androgenesis has different nutritional requirements than somatic embryogenesis (Hale *et al.*, 2022). Protocol development for new species and genotypes within responsive species continues to the present day, but slowly. There is more focus presently on understanding how protocols work in order to extend them to additional genotypes and species. Transcriptomic and epigenetic analyses of induced microspores have revealed some of the cellular and molecular responses required for or associated with androgenesis. For example, microRNAs appear to regulate early microspore responses to external stimuli; trichostatin-A, a histone deacetylase inhibitor, acts as an epigenetic additive; -phytosulfokine, a five amino acid sulfated peptide, promotes androgenesis in some species (Hale *et al.*, 2022). Additionally, present work on gene transfer and genome editing in microspores suggest that future endeavors will likely incorporate greater precision with the genetic composition of microspores used in doubled haploid breeding, thus likely to realize a greater impact on crop improvement (Sheng *et al.*, 2025). In this review, we evaluate basic breeding applications of androgenesis, explore the utility of genomics and gene editing technologies for protocol development, and provide considerations to overcome genotype specificity and morphogenic recalcitrance in non-model plant systems. Using landraces for broadening the genetic base of elite barley germplasm is hampered by heterogeneity and high genetic load. Production of DH line libraries can help to overcome these problems using different medium combinations and procedures (Table 3).

Table 3: Different media and pre-treatments for doubled haploid induction in barley

Media	Media composition	Pre-treatment method	Characteristics	Varieties	Reference
Cold pre-treatment method	Linsmaier & Skoog (LS) medium with growth regulators	Cold pre-treatment (4°C for 5-10 days)	Enhances microspore viability and embryogenesis	Barke	Kasha and Kao (1970)
N6 medium-based protocol	N6 medium with maltose and copper ions	Cold pre-treatment (4°C for 3-7 days)	Optimized for cereal crops, widely used in barley anther culture	Golden Promise	Chu *et al.* (1975)
Colchicine-induced chromosome doubling media	MS medium with colchicine and antioxidants	Direct colchicine treatment (0.05%-0.1%)	Used for converting haploids into doubled haploids	Lux	Barnabás *et al.* (1991)
Microspore culture media	FHG	Cold pre-treatment (4°C for 14 days)	Embryogenesis and green plant regeneration occurred in all genotypes	Twelve winter barley	Kahrizi *et al.* (2011)
Maltose-supplemented media	Modified N6 medium with increased maltose concentration	Mannitol pre-treatment (0.3M-0.7M)	Improved embryogenesis and green plant regeneration	Igri	Ohnoutkova *et al.* (2019a)
Western Australia DH development media	N6 medium with optimized hormone balance	Cold pre-treatment (4°C for 7 days)	Produces over 15,000 DH lines annually	Quench	Broughton *et al.* (2024)

6. Advancements in Anther Culture Protocols for Barley

Anther culture protocols for barley have undergone significant refinement to enhance haploid induction efficiency, green plantlet regeneration, and genetic stability in DH breeding programs. These protocols primarily focus on optimizing media composition, stress treatments, and genotype-specific responses to improve embryogenic potential and reduce albino plantlet formation. The key anther culture protocol and its modification (Lantos *et al*., 2022) in barley involves:

i. **Standard anther culture method**: This method involves cold pre-treatment of donor spikes to synchronize microspore development, followed by anther plating on induction media enriched with phytohormones (auxins, cytokinins) to stimulate embryogenesis. The success of this protocol depends on precise temperature regulation and genotype compatibility, ensuring optimal haploid embryo formation.

ii. **Modified stress-induced anther culture method**: To enhance microspore viability, this protocol incorporates heat shock or osmotic stress treatments, which trigger embryogenic responses in microspores. Cold shock (4°C for 3–7 days) and heat stress (32°C for 24 hours) have been shown to improve haploid induction rates, particularly in recalcitrant barley genotypes.

iii. **Ovary co-culture method**: This approach integrates ovary tissues into the anther culture system, providing additional growth factors and signalling molecules that enhance embryogenic response. The presence of ovary tissues reduces albino plantlet formation, improving the frequency of green plantlet regeneration.

iv. **Microspore isolation and culture:** A refined technique where isolated microspores are cultured in liquid media allows direct haploid embryo formation. This method eliminates the need for anther plating, improving embryo yield and uniformity while reducing somaclonal variation.

v. **Genotype-specific optimization:** Since barley genotypes exhibit variable responses to anther culture, tailored protocols adjust media composition, stress treatments, and phytohormone concentrations to maximize embryogenic efficiency.

7. Basic steps in anther culture for barley

i. **Donor plant selection:** To ensure that the barley plants are at the best stage of microspore growth, they are carefully selected for anther culture. The mid-to-late uninucleate stage is the best time to induce androgenesis since microspores at this stage have a high potential for embryogenesis. Choosing genotypes with high stress tolerance and regenerative capacity can increase the success rates of cultures.

ii. **Pre-treatment of anthers:** Anthers are subjected to particular stress treatments prior to culturing in order to promote androgenesis. Sugar starvation inhibits gametophytic development and promotes sporophytic pathways, while cold shock (mostly 4°C for several days) is frequently utilized to preserve microspore viability. Additionally, heat stress (32–35°C for 24–48 hours) can initiate embryogenesis. These treatments help maximize the efficiency of doubled haploid production (Fig. 2).

iii. **Anther isolation:** Using fine forceps or dissection tools anthers are removed from immature barley florets in a sterile environment. To prevent mechanical injury to microspores, which could lower the success of regeneration, this step must be done precisely. When handled carefully, contamination is reduced and viable microspores are kept intact for culture initiation.

iv. **Culture initiation:** To promote callus formation, separated anthers are put on induction medium that contains growth regulators like 2, 4-D or picloram. While activated charcoal lowers oxidative stress that may impede embryo development, other ingredients, such as copper sulfate, can improve the regeneration of green plants. To promote microspore reprogramming, the cultures are kept at regulated temperatures (22–24°C) in a dark environment.

v. **Callus or embryo formation:** Under favourable conditions, transition of microspores from gametophytic to sporophytic growth results in the development of direct embryos or callus. Albino plantlets, which are frequently the result of poor chloroplast development, are one of the main problems in anther production. Optimizing the medium's iron and magnesium concentrations has been explored to improve the regeneration of green plantlets.

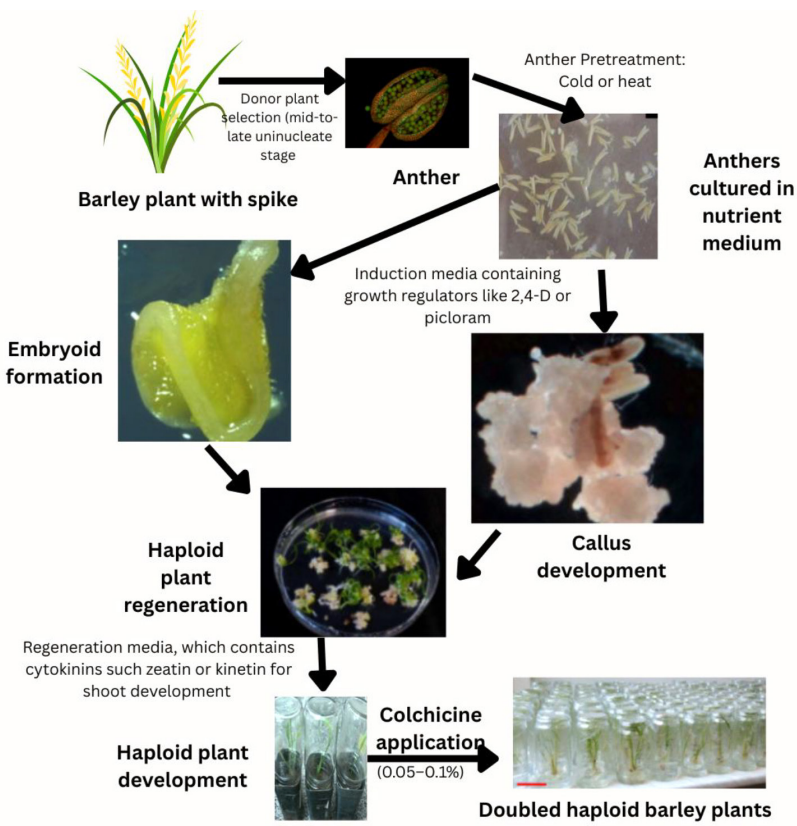

Fig. 2. A diagrammatic representation of anther culture technique in barley

vi. **Plant regeneration:** To promote shoot development, callus or embryos are placed in regeneration media, which contains cytokinins such zeatin or kinetin. Gibberellic acid is added to encourage elongation, and regeneration efficiency. Keep cultures at 22°C for a 16-hour photoperiod and before plants are moved to soil, successful roots and shoots are established.

vii. **Chromosome doubling:** In order to restore fertility, regenerated plants' chromosomes must be doubled because they are initially haploid. Colchicine (0.05–0.1%), which interferes with spindle formation during cell division and causes spontaneous chromosome doubling is frequently used to accomplish this. Some barley genotypes show natural chromosome doubling, reducing the need for chemical treatments.

viii. **Acclimatization and field trials:** Regenerated plants are moved to greenhouse or field environments for acclimatization after they have established a working root system. Their suitability for breeding programs is assessed based on agronomic features, genetic stability, and stress tolerance.

8. Factors Affecting Anther Culture

Doubled haploidy breeding is a powerful tool which requires the availability of reliable tissue culture protocols. In order to improve the regeneration efficiency in a wider range of genotypes, DH protocol needs to solve the problems like- low frequencies of embryo induction, plant regeneration, albinism, plant survival, genotype- and season-dependent response. There are several endogenous and exogenous factors which affects the embryogenic response of the cultured anthers (Atanassov *et al*., 1995; Datta, 2005; Wang *et al*., 2000) The factors that greatly affect the response of anthers to *in-vitro* culture are listed below:

8.1. Genotype

The genotype of the source material has a significant impact on the efficiency of DH plant production. DH induction effectiveness during specific androgenesis stages is known to be influenced by specific QTLs (Krzewska *et al*., 2012). In a study of a wide range of barley genotypes, Lazaridou *et al.* (2005) found that the average regeneration efficacy was 1.7 green plants per 100 anthers cultivated. The potential production of barley DH plants can be significantly reduced in the androgenic process, which can be due to the production of albino plantlets in different proportions depending on the cultivar. While the frequency of albino plantlets is negligible in certain genotypes (1%), it is nearly 99.7% in others (Caredda *et al*., 2000). Compared to spring cultivars, winter cultivars of barley have been reported to yield more green DH plants (Cistué *et al*., 1998; Makowska *et al*., 2015). While genotype dependence has been reported in barley, hundreds of DH populations have been generated and optimized protocols are possible for a broad variety of commercial varieties, breeding lines, and landraces (Broughton *et al*., 2014).

8.2. Pollen development stage

Pollen development stage strongly affects the success of anther culture. Different species reacts differently to the developmental window of embryogenic competence. In barley, the tetrad stage of microsporogenesis and the immediate post-first pollen mitosis are the most receptive phases for haploid embryo development. Microspores are most likely to transition from a gametic to a sporophytic developmental route during this phase. Study conducted by Guasmi et al (Guasmi *et al*., 2013) on different stages (early, mid-late, late) of culturing has highlighted their effect on androgenic response. Barley anthers are most responsive in culture when the majority of microspores are at the mid-to late-uninucleate stage (Han *et al*., 2020a).

8.3. Physiological state and growth conditions of donor plants

The DH production in barley is affected by the physiological conditions of the donor plants (Heberle-Bors, 1985), and also the other factors like endogenous levels of hormones and the nutritional status of the tissues of the anther. It has been observed by researchers that field grown plants are better in response compared to the plants of green house (Ercan *et al*., 2006). The androgenic response is strongly influenced by the donor plants' age and developmental stage. Better results are typically produced by plants that are in their best health and vigor. The anthers from the first flush of flowers in the season were found to be more responsive (Sunderland, 1971). Environmental factors that affect the quality of anthers collected for culture include temperature, light intensity, and nutrient availability.The environmental conditions such as the short days, low temperature have been reported totally unfavourable for the growth of plants (Heberle-Bors, 1985). The nitrogen status of plants

also greatly affects the yield of microspore embryos (Sunderland, 1978; Tsay, 1981).

8.4. Pre-treatment

Several pre-treatments are reported which influence the efficiency of DH induction (Table 4). Any physical or chemical pre-culture treatments applied to excised flower buds, excised anthers, and whole inflorescences before culture prevents the development of fertile pollen and act as a trigger for inducing the sporophytic (gametophytic) pathway. Factors like high temperature, high humidity, centrifugation, chilling, water stress, anaerobic treatment, sucrose and nitrogen starvation, ethanol, irradiation, microtubuli disruptive agents, electro-stimulation, high medium pH, heavy metal treatment are particularly studied for anther and microspore culture response. In barley both cold (SUNDERLAND and ROBERTS, 1979) and hot temperatures (Wilson *et al*., 1978) have been employed to the excised spikes and tillers.

Table 4: Different pre-treatment studies in barley anther culture

Cultivar	Pre-treatment	Inference	Reference
Igri	0.3 M Mannitol	More number of green plantlets were obtained	Hoekstra *et al.* (1992)
Igri	0.3 to 0.7 M Mannitol	Increase in the yield of green plants	Cistué *et al.* (1994)
Douchka Celinka Gotic	0.3 M Mannitol +10 mM $CaCl_2$	Induction of microspore embryogenesis	Li and Devaux (2001)
Igri Saida Libya	0.3 M Mannitol	Best results were obtained for embryo and green-plant production.	Guasmi *et al.* (2013)
Spring barley DH00-126	4 °C	Increase frequencies of morphogenic anthers and green plant regeneration	Bilynska (2020)

In order to induce *in-vitro* androgenesis, stress pre-treatments are necessary to re-programme the microspores' gametophytic pathway to the sporophytic one. A variety of stimuli, including osmotic and starvation stress (Castillo *et al*., 2014; Cistué *et al*., 1994) and a long-term (28 days) cold pre-treatment (Huang and Sunderland, 1982), can be employed to induce androgenesis in barley anther culture. Green plantlet development in barley anther culture was greatly increased by applying the Ficoll-400 and substituting maltose for sucrose (Devaux and Kasha, 2009; Kao, 1981). According to Makowska *et al.* applying gum Arabic accelerated the pace of plant regeneration (Makowska *et al*., 2015; (PDF) Gum Arabic influences the activity of antioxidant enzymes during androgenesis in barley anthers, 2024). Growth regulators play an important part in the induction of androgenesis as well. While some studies have employed hormone combinations 2,4-Dichlorophenoxyacetic acid (2,4-D), 2-Naphthaleneacetic acid (NAA), and kinetin to induce androgenesis in barley (Huang and Sunderland, 1982; Ohnoutkova *et al*., 2019a; Ohnoutková and Vlčko, 2020), 6-Benzylaminopurine is typically applied in induction media (Ohnoutkova *et al*., 2019b). In order to maximize the culture conditions and the productivity of green plant production, the interaction between Cu^{2+}, Ag^{2+}, and a pattern of DNA methylation has also been investigated during the induction of barley anther culture (Bednarek and Orłowska, 2020). Although cold pre-treatment is frequently employed, other techniques like mannitol exposure and colchicine treatment are being refined for improved outcomes. A comparison of different pre-treatment techniques in barley anther culture is shown below (Table 5):

8.5. Medium composition

The medium composition has been identified as a crucial factor influencing the androgenic response. The most commonly used basal media for anther culture are N6 medium (Chu *et al*., 1975), MS medium (Murashige and Skoog, 1962) and its modifications, Nitsch and Nitsch medium (Nitsch and Nitsch, 1969) and B5 medium (Gamborg *et al*., 1968), but there are many others. Generally, half-strength MS media is suggested for the

Table 5: Comparative analysis of different pre-treatment

Pre-treatment Method	Pros	Cons	Impact on Barley Anther Culture	Reference
Heat shock (32°C for 24-48 hours)	Stimulates microspore reprogramming, enhances embryo formation	Can cause excessive stress, reducing viability in some genotypes	Used in combination with other treatments to improve androgenesis	Huang and Sunderland (1982)
Sucrose pre-treatment (6%-12%)	Provides osmotic balance, enhances microspore survival	High concentrations can inhibit embryogenesis	Used to stabilize microspore development and improve embryo formation	Huang and Sunderland (1982)
Cold pre-treatment (4°C for 3-14 days)	Enhances microspore viability, improves embryogenesis, delays pollen maturation	Can increase albino plantlet formation, genotype-dependent response	Widely used for improving androgenesis, but requires optimization for different cultivars	Sunderland and Huang (1985)
Mannitol pre-treatment (0.3M-0.7M)	Improves embryogenesis, reduces stress-induced damage, enhances green plant regeneration	Requires precise concentration adjustments for different genotypes	Effective for low-responding cultivars, increases doubled haploid production	Cistué *et al.* (1998)
Colchicine treatment (0.05%-0.1%)	Induces chromosome doubling, accelerates doubled haploid production	Toxic at high concentrations, requires careful handling	Essential for producing homozygous lines, widely applied in breeding programs	Cistué *et al.* (1998)
Mannitol (62g/L) and cold treatment (4^0C)	Reduced albinism	Requires concentration adjustments	Improved green plant recovery	Sriskandarajah *et al.* (2015)
Dimethyl sulfoxide (DMSO) (0, 0.2, 1 and 2 % v/v)	Increase in green plants	Requires concentration adjustments	Increase in the number of green plants, leading to a twofold to fourfold increase in both cultivars and F_1 crosses	Echávarri and Cistué (2016)
Cold pretreatment at 4 °C	Enhances microspore viability, improves embryogenesis, delays pollen maturation	Can increase albino plantlet formation, genotype-dependent response	Varieties with high nitrogen use efficiency (NUE)	Xu *et al.* (2021)
0.7 M mannitol	Reduced albinism	Requires concentration adjustments	Green Plantlet Regeneration	Lantos *et al.* (2024)

Solanaceae (Gamborg *et al.*, 1968), and N6 medium for the cereals. By substituting maltose for sucrose as a carbon source, significant advancements have been made in barley anther development, resulting in increased rates of embryogenesis and improved regeneration of green plants. Research by Szarejko and Kasha Szarejko, (2003) demonstrated that replacing sucrose with maltose significantly increased the number of viable embryos in barley anther culture. Similarly, Lantos *et al.* (Lantos *et al.*, 2024) found that maltose enhances androgenic response and embryo formation. Nitrogen plays a crucial role in microspore development, and optimizing the balance between organic (amino acids) and inorganic (nitrate/ammonium) nitrogen has improved embryogenesis rates. Studies have shown that an appropriate nitrogen balance enhances microspore viability and reduces stress-induced abnormalities. Chu *et al.* (1975) investigated the role of nitrogen sources in anther culture and found that a balanced nitrogen composition significantly improved callus induction and plant regeneration. Agar was traditionally used as a gelling agent in induction media, but its limitations, such as inconsistent gel strength and nutrient diffusion, led researchers to explore alternatives. Ficoll and agarose provide a more stable matrix, improving embryo formation and plantlet survival. Kuhlmann and Foroughi-Wehr (Kuhlmann and Foroughi-Wehr, 1989)

reported that replacing agar with Ficoll or agarose increased the androgenic response and improved embryo formation. Additionally, Kao (1981) found Ficoll enhances microspore density leading to higher embryogenesis rates.

9. Inducing Doubling and Ploidy Level Determination

Chromosomes are doubled by nitrous oxide or colchicine treatments to create doubled haploids (Arshad *et al*., 2023). However, chromosome doubling is often achieved using an alkaloid colchicine and is an important step in DH development (Patial *et al*., 2015). Because colchicine breaks the spindle fibres during cell division, it hinders chromosomal segregation and results in cells having two sets of chromosomes. The effectiveness of this method depends on the haploid plant's physiological state, exposure duration, and colchicine dosage. DHs are produced when haploid plants go through this doubling process.•• The most effective treatment for doubling barley haploids in the early stages of development have been reported as colchicine plus dimethyl sulfoxide (DMSO) (Kasha, 2005). Chromosome doubling procedures have been improved by researchers to increase success rates while minimizing tissue damage and toxicity.

Doubling of the haploid genome doubles the DNA content in the nucleus. Different methods can be used to assess the ploidy level (Table 6). A traditional cytogenetic method for figuring out how many chromosomes a cell contains is chromosome counting. Chromosomes are stained and examined under a microscope, usually when they are at their most condensed stage during metaphase. This technique is crucial for detecting chromosomal abnormalities and verifying ploidy levels. Even though it requires extensive work, it is nonetheless a trustworthy method for genetic and breeding studies. Other technique is chloroplast scoring in stomatal guard cells of the lower epidermis of young leaves (Detrez *et al*., 1989). For chloroplast scoring, the leaves are fixed in ethanol: acetic acid (3:1, v/v) and stained with potassium iodide solution. Counts are then established from a minimum of 25 stomata in each leaf analysed and a minimum of 3–5 leaves are examined. Novel method to assess the ploidy level through the analysis of cellular DNA content is flow cytometry, which is a very fast and reliable method (Garavello *et al*., 2019). In flow cytometry, a parameter is a measured property of the particles, frequently assimilated to an optical channel, whereby a one-parameter histogram will plot the subpopulation of cells that contain a given quantity of DNA or bind a given number of antibody/ fluorophore molecules (McKinnon, 2018). 4,6-diamidino-2-phenylindole (DAPI) (AT-specific) is the most frequently used dye to determine ploidy level by flow cytometry, while propidium iodide (PI), usually believed to be non-base pair specific but which nevertheless exhibits a GC-preference (Vinogradov, 1994), is preferred for the determination of genome.

Table 6: Method used for ploidy level determination

Method	Principle	Advantage	Limitations	Reference
Flow Cytometry	Measures DNA content using fluorescence intensity in nuclei stained with dye	Rapid, accurate, high throughput	Requires expensive equipment and trained personnel	Garavello *et al.* (2019)
Chromosome Counting	Microscopic observation of chromosomes in dividing cells	Direct and precise	Time-consuming, technically demanding, limited to dividing cells	Littlejohn and Pienaar (1988)
Stomatal Size Measurement	Indirect method based on correlation between cell size and ploidy	Simple, non-destructive	Indirect and not highly reliable	Domblides *et al.* (2022)

10. Haploidy as a Barley Breeding Tool

The development of crop cultivars by crossbreeding is both time and resource consuming. It takes between 7 to 8 years from the time the cross is made until phenotypically advanced uniform lines are produced. These are then evaluated for at least 3 years to identify potential candidate lines for cultivar release. The continued demand for new cultivars with specific characteristics requires accelerated methods in plant breeding for the development of new cultivars. Table 7 highlights potential differences between DH and conventional breeding program. The doubled haploid technology has been used in different plant breeding and genomics research for accelerated breeding. The use of DH technology integrated with advanced

technologies is fastening the research and varietal development program. MAS can be used as an indirect selection method to speed and increase the precision of the genetic progress, reduce the number of generations, and when integrated into optimized molecular breeding strategies, it can also lower the costs of selection (Dwivedi, 2007). Thus, integrating MAS with DH provides new opportunities for the development of improved selection methods that maximize selection gains and accelerate development of crop cultivars.

Table 7: Comparison of doubled haploidy and conventional breeding approach

Particulars	Conventional breeding	Doubled Haploidy	Reference
Time required for cultivar development	7-8 years or more	2-3 years	Hale *et al.* (2022a)
100% homozygosity achievable	Not possible	Possible	
Fixation of heterosis	No	Yes	Badu *et al.* (2017)
Cost	Less	More	Patial *et al.* (2023b)
Recessive mutants identification	Difficult	Easy	Hale *et al.* (2022a)

Doubled haploids provide a unique opportunity to create a large number of fixed recombinants simultaneously, allowing selection within the same time. Compared to gynogenic methods, where the total number of recombinants from a single event is limited, the androgenesis system (microspores) provides the flexibility to generate the large number of recombinants (Hale *et al*., 2022).

Barkley and Chumley (Barkley and Chumley, 2012) compared the conventional and DH wheat variety development, and predicted that a 150% increase in yield potential may be achieved with the use of DH technology. *In-vitro* androgenesis techniques, such as anther- and isolated microspore cultivation, are widely used in barley breeding and research initiatives. Because of its high production of embryo-like structures and green plantlets production, barley is regarded as a model cereal crop for DH plant development *via* androgenesis (Cistué and Echávarri, 2021). Additionally, a number of additional technological techniques, such as genome editing (Han *et al*., 2020b), induced mutations (Vagera *et al.* 2004), genetic transformation (Cistué and Echávarri, 2021), and marker-assisted selection (Dwivedi *et al*., 2015), have effectively merged these approaches. Wessels and Botes (Wessels and Botes, 2014) showed that integration of MAS and DH technology into conventional breeding processes could increase the speed of cultivar development. Large numbers of inbred lines are attained at once in a DH-based breeding pipeline compared to multiple years and stages in a conventional method. This eliminates the need for handling larger numbers of breeding materials from different generations of inbreeding. In otherwords, significant field resources are saved by allowing smaller population sizes to produce a homozygous (fixed) trait, and/or evaluation of better performing lines (Park *et al*., 1976). Large genetic distances of DH lines derived from landraces help to broaden the genetic base of the germplasm (Böhm *et al*., 2017). By generating DH lines from these landraces, breeders can efficiently transfer novel alleles into elite breeding populations without the genetic segregation that occurs in conventional breeding. Due to the low population structure and rapid decrease of linkage disequilibrium within populations of DH lines landraces, these would be an ideal tool for association mapping. Ohnoutkova and Vicko (Ohnoutková and Vlčko, 2020) reported homozygous transgenic barley plants by anther culture and obtained transgenic homozygous DH lines from six different transgenic events by using anther culture. Anthers were isolated from T0 transgenic primary regenerates and cultivated *in vitro*. The application of these methods enables rapid evaluation of transgenic lines for gene function studies and trait evaluation.

10.1. Integrating doubled haploid technology with backcross breeding and marker-assisted selection for precise allele introgression in barley improvement

When used in conjunction with marker-assisted selection (MAS), DH technology significantly improves the effectiveness of backcross breeding by facilitating accurate allele introgression into elite barley cultivars (Collard and Mackill, 2008). Conventional backcross breeding involves lengthy breeding cycles since it takes several

generations to stable desired alleles while removing undesired characteristics. But because DH lines are fully homozygous, introgressed alleles can be fixed quickly in a single generation, greatly cutting down the time needed to establish genetic uniformity (Ren *et al*., 2017). In order to ensure the precise selection of DH lines carrying target genes for stress resilience, disease resistance, and yield stability, MAS further refines this procedure by employing genetic markers associated with advantageous features. Breeders can increase selection precision by combining DH technology with MAS to effectively remove undesirable genetic background and speed up the recovery of recurrent parental genomes (Gunundu *et al*., 2023). By optimizing the effectiveness of trait selection in barley breeding programs, this combination not only expedites allele introgression but also increases genetic gain.

10.2. Molecular integration in doubled haploid breeding

Modern breeding techniques have greatly benefited from the combination of genomic selection, MAS, and QTL mapping; yet, their use in DH technologies for barley improvement is still neglected (Jiang *et al*., 2025). Because DH lines are fully homozygous, they offer a special chance for precision genetic selection, enabling precise trait mapping and quicker breeding cycles. Integrating high-throughput genotyping, genome-wide association studies (GWAS), and MAS can greatly improve the efficiency of DH breeding, especially when it comes to identifying elite lines that carries disease resistance, yield stability, and abiotic stress tolerance (Xu *et al*., 2017). Breeders can identify advantageous alleles linked to important traits by using high-throughput genotyping, which provides large-scale genetic data and enables quick screening of DH populations. Combining this with GWAS makes it easier to pinpoint genotype-phenotype relationships and find genetic loci associated with improved stress tolerance and consistent yield performance (Nouraei *et al*., 2024). Furthermore, MAS techniques target certain genes that control drought tolerance, disease resistance, and nutrient-use efficiency, streamlining the selection process with the use of established molecular markers. Breeders can ensure that only high-performing DH lines progress in breeding programs by combining these strategies, which can speed up trait introgression, improve genetic accuracy, and shorten the breeding cycle (Sinha *et al*., 2023). Additionally, by combining DH technology with predictive breeding frameworks and machine learning algorithms, selection efficiency can be maximized. The applications of different molecular approaches in DH breeding are highlighted in Table 8.

Table 8: Key molecular approaches for enhancing genetic selection and trait improvement in barley doubled haploidy breeding

Molecular Approach	Application in DH Breeding	Advantages	References
Genomic Selection (GS)	Predicts breeding values of DH lines using genome-wide markers	Accelerates selection, improves trait prediction	Budhlakoti *et al.* (2022)
Marker-Assisted Selection (MAS)	Uses molecular markers linked to desirable traits for DH line selection	Enhances precision, reduces breeding cycles	Collard and Mackill, (2008)
QTL Mapping	Identifies genomic regions associated with key agronomic traits	Facilitates trait introgression in DH populations	Kim *et al.* (2004)
Genome-Wide Association Studies	Detects genetic variants linked to complex traits in DH populations	Provides insights into genetic diversity and trait inheritance	Chaikam *et al.* (2019)
Haplotype-Specific Markers	Identifies favourable haplotypes for trait improvement in DH lines	Improves selection accuracy and genetic stability	Weber *et al.* (2023)
Predictive Breeding Models	Uses machine learning and statistical models to optimize DH selection	Enhances efficiency and reduces experimental costs	Cheng and Wang, (2024)

11. Integrating CRISPR/Cas9 and Anther Culture for Precision Doubled Haploid Line Production

Doubled haploid production in barley is being revolutionized by the combination of CRISPR/Cas9 gene editing with anther culture, which improves genetic precision and speeds up breeding cycles (Impens *et al*., 2023). Breeders can alter important genes that cause haploid induction, spontaneous chromosomal doubling, and stress tolerance by using CRISPR/Cas9, which enables targeted genome alterations and ultimately increases DH efficiency. Notably, haploid induction rates can be increased while undesirable albino plantlets are eliminated by CRISPR-based modifications in centromere histone H3 (CENH3) genes (Kuppu *et al*., 2020). Additionally, CRISPR-based methods minimize the need for chemical chromosomal doubling agents like colchicine by enabling precise genetic alterations that maximize spontaneous haploid induction (Sheng *et al*., 2025). Higher rates of green plantlet regeneration result from CRISPR-induced changes in chloroplast-associated regulatory genes, which aid in the removal of albino plantlets, which are frequently linked to impaired plastid development (Erdoğan *et al*., 2023). To further improve DH breeding techniques in barley, multiplex CRISPR editing provides a potent tool for altering several loci linked to stress tolerance, disease resistance, and yield stability in addition to haploid induction (Chen *et al*., 2024). The capacity to precisely tune genes linked to haploid induction, stress tolerance, disease resistance, and yield stability in barley has made multiplex CRISPR/Cas9 editing a potent tool for altering numerous genomic loci at once. Multiplex CRISPR dramatically speeds up trait improvement while preserving genetic integrity by enabling targeted alterations across many genomic areas, in contrast to traditional breeding or single-gene editing techniques (Lorenzo *et al*., 2022). Multiplex CRISPR editing permits simultaneous alterations in dehydration-responsive element binding protein and heat shock factor genes, boosting drought and heat tolerance in DH lines (Chakraborty and Wylie, 2025). By fine-tuning abiotic stress-responsive pathways, CRISPR-edited DH plants demonstrate increased water-use efficiency, root architecture, and osmo-protectant accumulation, making them more tolerant to climate fluctuation (Sami *et al*., 2021). By using multiplex CRISPR editing to target the mildew resistance locus (HvMLO) and pathogen defense-related genes (HvPR1, HvRPM1), resistance to rust, powdery mildew, and fusarium head blight can be increased (Waites *et al*., 2025). Breeders can layer numerous resistance genes using this method, guaranteeing broad-spectrum disease protection without sacrificing potential production.

12. Challenges and Prospective Solutions for Enhancing the Efficiency of Barley Anther Culture

Number of challenges restricts the effectiveness and broad use of barley anther culture (Table 9). Large-scale production may be made easier by incorporating automation into anther culture activities, which would boost productivity and cut expenses.

Table 9: Challenges and Prospective Solutions for Enhancing the Efficiency of Barley Anther Culture

Challenges	Future Directions
Genotype dependency is one of the main challenges because different barley genotypes have a varying androgenesis success rate which makes standardization methods difficult.	By addressing genotype-specific needs, research into more customized and enhanced media formulations may increase the effectiveness of plant regeneration and embryogenesis. The identification of molecular markers linked to androgenesis might allow for more accurate selection of responsive genotypes. Barley genotypes with improved androgenic responsiveness could be created by genetic engineering, guaranteeing greater success rates in anther culture technique. To overcome genotypic limitations, identifying and modifying important regulatory genes by combining transcriptomic, proteomic, and metabolomic methods with genome editing tools like CRISPR/Cas systems should be integrated. Furthermore, the resilience and scalability of DH generation across a variety of barley germplasm may be further improved by the creation of genotype-independent induction techniques and innovative culture conditions.

Another frequent problem, particularly with cereal crops like barley, is the development of non-viable albino plantlets.	By improving stress pre-treatments like cold shock and heat exposure to boost embryogenesis and optimizing growing conditions, the controlled environment technique dramatically increases the rates of green plantlet regeneration.
Despite improvements in media compositions and procedures, several cultivars show limited rates of embryogenesis and regeneration.	Elucidation of the molecular and epigenetic processes controlling microspore totipotency and embryogenic transition should be the top priority of future studies, with a focus on genotype-responsive pathways.
The viability and development of the cultivated anthers and microspores are impacted by their extreme sensitivity to environmental stressors.	Future research should concentrate on understanding the molecular stress-response networks and identifying important protective regulators, such as heat shock proteins, antioxidants, and stress-induced transcription factors, in order to mitigate the negative effects of environmental stress on microspore and anther cultures. Furthermore, cellular resilience and embryogenic potential may be improved by the introduction of exogenous signaling molecules (such as polyamines and phytohormones), optimized *in-vitro* culture microenvironments, and the implementation of controlled pre-treatment regimens.
Although colchicine remains the most commonly employed antimitotic agent for inducing chromosome doubling in haploid plants, its application necessitates precise calibration, as excessive concentrations or prolonged exposure can result in cytotoxicity, tissue necrosis, and reduced plant viability.	Future studies should focus on maximizing the effectiveness of chromosomal doubling while decreasing phytotoxic effects by optimizing colchicine dosage, exposure time, and delivery mechanisms. There are encouraging prospects for safer and more efficient polyploidization through the investigation of substitute, less harmful antimitotic substances, such as trifluralin and oryzalin. Additionally, improvements in targeted delivery methods (such as localized application methods or nanocarriers) may improve tissue-specific uptake and lessen systemic toxicity.

Future developments will probably concentrate on combining machine-learning models and genomic selection techniques to improve haploid induction efficiency even more, enabling precision-driven and sustainable barley breeding.

Conclusion

Double haploid plant production methods are widely used in crop breeding and research programs because of their ability to produce genetically pure lines in one generation. Androgenesis in barley has been successfully exploited for more than 5 decades and many haploid and doubled haploid cultivars/lines had regenerated for basic and applied research. Despite challenges such as genotype dependency and variability in success rates, this technique has been widely used to develop new crop varieties and improve traits. Barley is now considered to be one of the model crop species, particularly in cereals, for research on the identification of genes involved in microspore embryogenesis induction and for genes involved in embryo development stages. As a diploid species, barley is frequently selected for biochemical and cytological investigations of microspore androgenesis. The effectiveness and application of DH technologies have been greatly improved by advanced molecular technologies. Methods like CRISPR-based gene editing, genomic selection, and MAS have helped in precisely identify and modify genetic characteristics in haploid and DH lines. By enabling the quick screening and early selection of potential genotypes, these technologies greatly shorten the breeding cycle. The fixation of elite alleles in barley has been enhanced by the integration of molecular markers with DH production systems, which has also speeded up the generation of high-yielding, disease-resistant, and stress-tolerant varieties. Additionally, molecular tools have helped in the optimization of haploid induction and chromosome doubling methods as well as the study of the genetic regulation of androgenesis. Collectively, these technological advancements highlight the indispensable role of DH methodologies- augmented by cutting-edge molecular innovations- as integral components in the acceleration and refinement of contemporary barley genetic.

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Author contributions

The review was written and enriched by MP and SKB. All authors read, edited, and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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No

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