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Review Article

Molecular markers applied to genetic diversity analysis and genome - wide association studies for micronutrients in grains and biotic stresses traits in barley (*Hordeum vulgare L.*)

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ABSTRACT

The cultivated barley (*Hordeum vulgare L.*) ranks the fourth most important cereal worldwide. It feeds animals, produces malt, and is used in the human diet. Yield increase and yield stability are the top barley breeding goal. However, diseases such as the Net form of Net blotch (NFNB) and powdery mildew (PM) reduce yield and grain quality. For barley destined for human consumption, micronutrients increase in grains, especially zinc and iron, is essential to alleviate malnutrition. Thus, breeders must select new loci and use them to develop higher-yielding, nutritious, and disease-resistant cultivars. This study reviews the importance of genetic diversity analysis using molecular markers and Genome-wide association studies (GWAS) in barley breeding. Genetic diversity studies are crucial for conservation and utilization of barley germaplasm in plant breeding. Secondly, we discuss genome-wide association study (GWAS) uses to locate genomics regions associated with important barley traits such as disease resistance to NFNB and PM, and micronutrients (Zn and Fe) content in grains. Significant SNP identified in GWAS studies once validated in other experiments or populations, they can be converted into user-friendly markers and used to develop barley cultivars with improved quality, and disease resistance via marker-assisted selection.
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1. Introduction

Plant breeders must increase food production to feed the increasing world population. At the same time, climate change has negatively impacted agriculture productivity by elevating principal abiotic and biotic stresses. Climate change and increased food requests represent a considerable threat to food security. Even though only around 5% of produced barley is used for human food, in some regions such as North Africa, Ethiopia, and the Andes, it constitutes a staple crop, used mainly in making bread. Barley remains an economically important crop, ranking fourth cereal in production and importance worldwide after wheat, maize, and rice. A large percentage of barley feeds livestock as a source of calories, around 70 %. It is the main ingredient in beer and whiskey production; about 20% to 25% goes to malt, contributing to the economic increase [1]. Moreover, barley adapts to various conditions and tolerates abiotic stress more than other cereals [2]. Therefore, it is often cultivated in marginal regions.

Barley varieties cultivated in many regions, especially marginal regions, have low yields due to poor culti-

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vars and disease susceptibility and are lower in micronutrients. Thus, the need to breed cultivars with high yield, nutritious, abiotic, and biotic stress tolerance to provide poor farmers with suitable cultivars. It will improve the barley productivity and the lifestyle of farmers and barley breeders. The overall objectives of this review are to illustrate how to enhance barley productivity using genomics tools. We review in this study the importance of genetic diversity analysis in barley germplasm and the importance of GWAS in identify genomic loci associated with important barley traits.

Barley is a diverse crop that adapts to different climatic conditions. However, domestication and breeding have reduced the diversity of modern cultivars, and they are more vulnerable to abiotic and biotic stress. Access to diverse germplasm to select from can benefit breeders to improve and develop improved cultivars since breeders use it for crossing or finding new alleles. In addition, genebanks hold raw materials to improve traits; they need to be characterized for better exploitation and conservation [3]. Thus, estimating barley diversity and introducing new sources of variation in breeding programs will prevent crop vulnerability to many stresses caused by climate change. It will also help improve other traits.

Most traits of agriculture's importance, such as yield, disease resistance, and micronutrients, are quantitative traits. Yield increase and stability are the top goals for barley breeding. However, disease and biotic stress reduce yield and grain quality. On the other hand, micronutrients in the body participate in several functions; their absence leads to malnutrition, which is a severe public health crisis, especially in rural areas. Increasing micronutrient contents in staple crops will help alleviate malnutrition. Breeding for these traits is challenging since they are affected by many loci with minor effects and environments. Their improvement requires mapping genomic loci associated with these traits, known as quantitative trait loci (QTL) which can be incorporated into breeding to develop improved cultivars.

QTL mapping is a statistical approach aiming to detect the association between phenotype data and genotype to locate markers or QTL associated with a given trait. QTL can be identified via GWAS or linkage mapping; both approaches have the same aim but differ in some properties. The main difference is that linkage mapping uses created populations from crossing divergent parents. In contrast, GWAS or association mapping uses natural and diverse populations. GWAS has become a more popular approach than linkage mapping. It is fast since there is no time to create a population. Its results apply to the entire germplasm, and more alleles can be tested [4].

Once marker-trait associations (MTA) are identified and validated, they can be used in breeding via MAS or genomic selection. Recent genomics advances observed in barley breeding, such as a high-quality reference genome, SNP genotyping at high throughputs, and biostatistics tools, have made mapping genomic loci (QTL) associated with important barley traits accessible to barley breeders and geneticists [5]. In this context, this review shows the importance of marker traits associated (MTA) with important traits in barley from GWAS such as yield-related traits, net blotch, powdery mildew diseases and zinc and iron content in grain. Identified markers are validated in other experiments, they can be applied in breeding. For instance, they can be used in marker-assisted selection for the final goal of increasing the yield of cultivars, breeding for NFNB and PM resistance, and developing biofortified barley rich in zinc and iron.

2. Barley

2.1. Importance and genetics

Cereals constitute staple foods worldwide; they provide carbohydrates, protein, and other nutrients for human and animal diets [6,7]. The cultivated barley (*Hordeum vulgare L. ssp. Vulgare*) ranks the fourth most important cereal worldwide after wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and rice *(Oryza sativa* L.). The majority of barley, around 70%, is used to feed animals. The 25% goes to malt for producing alcoholic drinks (beer and whiskey), contributing to considerable economic growth. The rest, around 5%, goes into the human diet. It constitutes a staple crop in some areas, such as North Africa and Asia [8].

Barley consumption for humans is projected to increase due to its health benefit. It is rich in β glucan, offering several health benefits such as lowering blood pressure, increasing satiety, and reducing heart disease risk. In addition, 100g of barley contains 334 Kcal of energy,10.6g of protein, 2.1g of total fat, 60.8 g of carbohydrates,14.8g of fibers, 50 mg of Calcium, 6mg of iron, and 0.8 mg of zinc [9]. Barley's end uses dictate which quality trait breeders will target. The main target traits for barley destined for human food include hulless barley, an increase of β glucan, taste, and micronutrients such as zinc and iron. In comparison, malt barley uses primarily hulled and 2-row barley due to its large size compared to six rows. The protein content is another essential for malt barley [10].

Barley inflorescence is in the form of a spike, with

the rachis representing the central axis. The rachis contains nodes and internodes. The floret (spikelets) is attached to the nodes. At each node, there are three spikelets. For two-row barley, the center spikelet is the fertile one. In contrast, all three florets are fertile in 6-row barley [7].

Barley is considered a crop model for other crops, mostly the tribe of Triticeae, in four key areas. First, in the Fertile Crescent, barley was domesticated. Through this, other crops places of domestication were drawn. Secondly, in plant disease research, disease resistance loci to powdery mildew (PM). The mutation locus conferring resistance to PM, the mildew resistance locus o known as MLO locus, was first found in Ethiopian barley landraces. PM is found in about ten thousand plants, and the *mlo* loci can be applied to control PM in other plants [11]. Thirdly, barley is also a model in genetic studies, such as mutation breeding, since it is a self-pollinated and diploid (2n=14) crop with a low chromosome number. Furthermore, it was among the first crop where the mutation started in 1928 [12], and extensive barley mutant collections are available. Fourthly, it is also considered a model crop for adaptation to diverse climates conditions since it tolerates abiotic stress more than other cereals [2,8]. The barley gene *HVA1* on 1H expressed in mulberry, rice, wheat, and oat confers their transgenic plant's tolerance to low temperature, salinity, and drought stresses than checks lines [13,14]. Additionally, barley matures early than wheat and requires less input (fertilizers). It is found in marginalized regions where wheat and other cereals cannot survive.

The cultivated barley (*Hordeum vulgare L. ssp. Vulgare)* is a self-pollinated crop, diploid, with seven pairs of chromosomes 2n=14. Its genome size is approximately 5.1Gbp, with 39,734 high-confidence genes. In 2012, International Barley Sequencing Consortium [15] published the first genome of the barley cultivar Morex. Then in 2017 [16] released the highest quality barley map. However, some alleles are absent in the reference cultivar. Barley pangenome aims to include sequences of diverse germplasm (20 lines were selected) to capture more diversity. In addition, it will offer more usage in genomics breeding for trait improvement [17].

2.2. Diversity in barley germplasm

Barley belongs to the Triticeae tribe, the Poaceae family, Genus *Hordeum,* with 33 species. The number of barley accessions genre *Hordeum* available in genebanks was estimated at 453,602, with the majority the cultivated barley, followed by its closest wild relative *Hordeum spontaneun.* These accessions represent a source of new genes and alleles that can be exploited in breeding to improve traits of agriculture's importance [18]. Leading barley gene banks holders are Plant Gene Resources of Canada (PGRC) with 40031 accessions, United States Department of Agriculture (USDA) with 29874 accessions, Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA, Brazil) with 29227 accessions, and the International Center for Agricultural Research in the Dry Areas (ICARDA) with 26679 accessions [18].

Screening all these accessions in the gene bank can be tedious. Therefore, breeders have established core collections to monitor and exploit barley diversity [19]. The core collection assembles a set of genotypes representing a large diversity in a crop, both cultivated, genetic stocks, and wild barley. In addition, allele mining tools such as focused identification of germplasm strategy (FIGS) will accelerate the selection of new alleles. FIGS tools entail screening a set of germplasm found in the regions where the trait is challenged. For example [20] studied Net blotch resistance in barley using FIGS.

Barley germplasm includes landraces, cultivars, genetics stocks, breeding and research materials, and wild barley. Landraces are native lines selected by farmers for generations; they result from spontaneous mutation and natural outcross and contain enough genetic variation. Wild relatives include wild forms of the species of barley. The cultivated barley *Hordeum vulgare L. ssp. vulgare* originated from its wild form *Hordeum. vulgare subsp. spontaneum or Hordeum spontaneum* is the closest to the cultivated barley, followed by *Hordeum bulbosum* and other wild relatives. Wild relatives of barley show large genetic diversity than cultivated barley. Cultivars are pure, uniform, and stable for many years; they include commercialized lines. Genetic stocks result from mutations for genetic study and breeding materials. Landraces and wild barley are the primary sources of new genes for breeding disease resistance, yield, and other traits [18].

Barley is divided into three gene pools which show how closely related and the result of their crossing. *Hordeum vulgare subsp. vulgare* and *H. vulgare subsp. spontaneum* belong to the primary gene pool. Crossing of germplasm in this pool is easily made. In contrast, the second gene pool contains only *H. bulbosum*. This second pool's accession is not easily crossable with the primary gene pool by sexual reproduction, and offspring shows low fertility. The tertiary gene pool contains the remaining species; gene transfer from this pool to the primary gene pool requires genetic transformation techniques such as embryo rescue [21].

In barley and other self-pollinated crops, making crosses is one of the ways to increase genetic diversity. DNA Recombination and reshuffling of alleles during meiosis increase genetic variation, which is responsible for different adaptability to environmental change. Mutation breeding and polyploidisation are alternatives methods to increase barley genetic diversity. Barley is among the main crop where mutation breeding started. Induced mutation started with x-rays, then neutrons, and progressed to chemicals [22].

3. Molecular markers in barley breeding

3.1. Evolution of molecular markers

In barley breeding, molecular markers have evolved from protein-based markers (isoenzymes) to next-generation sequencing NGS-based markers. Isoenzymes or protein-based markers were the first markers used in barley breeding; however, they are low polymorphisms and hard to detect [23]. The discovery of DNA-based markers improved accuracy and replaced the use of isoenzymes markers. Restriction fragment length polymorphisms (RFLP) markers detect variation in DNA fragment length after restriction enzymes digest DNA. RFLP is a gel base marker, constitutes a laborious technique, and requires a large amount of DNA. An example of their application was in the study of [24] to select loci associated with resistance to cereal cyst nematode.

Next were the polymerase chain reaction (PCR) based markers. They improve DNA assessment since they are more sensible, automatic, and reliable and require less DNA quantity. RAPD, AFLP, and SSR markers are primarily used in barley breeding. The Random amplified polymorphic DNA (RAPD) markers are also gel-based, tedious, and have less replicability between laboratories.

Barua et al [25] used them to find markers linked to *Rhynchosporium secalis* resistance. On the other hand, the amplified fragment length polymorphisms (AFLP) markers are also PCR-based, better than RFLP, and more reproducible [26]. Simple sequence repeats (SSR) or microsatellite markers are polymorphs abundant in the genome and transferable among the population; they have been widely used in barley breeding, for instance, for genetic diversity analysis [27].

The SNP marker's discovery completely changes the game in barley breeding; they are frequent in the genome and accessible at high throughput for barley genotyping. The low cost of genotyping and advances in bioinformatics make them the most used for crop improvement. Illumina Infinium assay and Affymetrix GeneChip represent the most used assay platform for high-throughput genotyping [28].

Several SNP platforms exist for Illimuna Infinium and are used for barley characterization and locating genomic regions associated with the trait. For instance, the Illimuna barley oligonucleotide pool assay1(BOPA1) and BOPA2 include 1536 SNP [29]. [30] upgraded it to a 9K SNP platform with 7,842 SNP, of which 6,094 SNPs have known positions. Additionally, [31] developed the 50 K SNP array, which offers higher quality and coverage; it contains 44,040 SNP with 29,415 annotated genes. However, the SNP array is designed from a limited set of populations, called the discovery panel. Ascertainment bias may occur when analyzing another population, for example, wild populations [10,32]. Rare SNPs are often not discovered, and SNPs absent in the discovery panel will not be added to the assay [33].

NGS-based markers are more throughput than other markers. They entail many protocols and can be differentiated by their use or not of restriction enzymes to reduce genome complexity. Genotyping-by-sequencing (GBS) is the most used in barley. However, it is still costly and requires computer and bioinformatics skills [34,35]. GBS provides high-quality data; suited for genotyping when SNP information is unavailable. It uses restriction enzymes to reduce DNA complexity [28,36].

3.2. Advantage of molecular markers

Molecular markers offer several advantages over phenotype selection. They offer more precise selection, increase accuracy, therefore, more genetic gain. They are effective for selecting a challenging trait, such as disease traits whose expression depends on the growth stage (adult plant stage) and environment. Moreover, they can be cheaper than phenotyping and show the possibility for large-scale sample analyses, resulting in reduced time and cost for selection, which increases selection intensity. In contrast, morphological and biochemical markers expression depend on plant stage and the environment [37].

Selection based on molecular markers has advanced barley breeding since it exploits the genetic inheritance of the desired trait. Markers linked to desired gene or QTL are used to screen lines with the desired trait. For instance, MAS is used to speed the breeding and improve the selection of desired traits [28,38]. Good molecular markers are the ones linked to the gene that affects the trait of interest and segregates with that trait, are polymorph, high throughput, abundant in the genome, low cost, reproducible, codominant, and easy to use [39].

In barley breeding, molecular markers are used in several areas, including analysing genetic diversity, determining relationships among accessions, characterizing plants, and pyramiding genes. Moreover, they are used for genotyping, which can be applied for MAS, QTL mapping (GWAS and linkage mapping), and genomic selection GS [28].

4. Genome-wide association study GWAS

The most important traits in barley breeding are affected by many loci (quantitative trait loci QTL). QTL mapping allows to identify and locate genomic loci (marker-trait association MTA and QTL) associated with the quantitative trait on their specific chromosomes in order to apply the marker in breeding for the trait improvement, e.g.via, marker-assisted selection (MAS) [40].

QTL mapping uses a statistical model on phenotype and genotype data to locate chromosome regions that contain QTL loci that affect phenotype traits. The low cost of high-throughput genotyping with the SNP array platform and high-quality reference map has made QTL mapping accessible to barley geneticists [41]. GWAS, also known as linkage disequilibrium mapping and biparental mapping (linkage mapping), are used tools for QTL mapping. They have the same aims but differ in some properties (Table1) [32,42,43].

Table 1. Difference between GWAS and Genetic Linkage Mapping.

Aspect	**Linkage mapping**	**GWAS**
Population	Biparental crosses such as double-haploid (DH), back-cross BC, F2 populations, and recombinant inbred lines (RILs) populations; thus, require more time	A natural and diverse population, less time is required.
Number of alleles tested	Low alleles (consider two alleles)	More alleles
Resolution	Low resolution due to less recombination	Higher resolution due to recombination over generations
Falses positive	Fewer falses positives observed	More falses positives due to population structure
Quantity of marker	Less marker is required since few alleles are considered	More markers are required
Results	Application is limited to the mapping population used	Applicable to the whole population

These differences have made GWAS more popular than biparental mapping. GWAS started in animal and human studies. In-plant science, GWAS started with maize (*Zea mays L.)* and *Arabidopsis*, then with other crops, including barley [4].

4.1. Factors affecting GWAS

The power of GWAS analysis is affected mainly by sample size, phenotype and genotype data quality, statistical analyses, population structure, allele frequency, and linkage disequilibrium. Large and diverse samples offer more information and more power. About 100 to 500 samples gave good results. Wang et al demonstrated that above 384 samples are statistically required for GWAS studies to detect MTA [44]. Clean and accurate phenotyping is essential for a good GWAS. GWAS requires an accurate phenotype of a trait with high heritability. Plant scientists use tools such as image device analysis, robotics, and sensors to assess high-throughput phenotyping. Good genotype data from GBS or SNP assay, such as Illumina 50k for barley, are required on an excellent GWAS, where failed SNP and samples are removed from the analysis.

Population structure can cause spurious association, which is an association based on structure rather than genes and trait association. They need to be corrected to reduce falses associations; their effect has to be removed from the genotype matrix. The remaining SNP value represents the biological effect. Therefore, a robust statistics approach that corrects population structure and relatedness to reduce falses associations is required [4]. Barley population is mainly structured according to row type, growth habit, geographic origin, and end-uses.

Software to estimate population structure includes

STRUCTURE [45], which estimates the number of subpopulations K. FastStructure [46] infer population structure from a large SNP data set. Additionally, Principal Component Analysis PCA corrects population structure by reducing the dimension of genotype data [47]. Plink software plink software [48] estimate population structure from a large dataset [49]. Moreover, kinship (pairwise relatedness) reduces falses positives [50]. Therefore, a mixed linear model (MLM) using kinship and population structure is among the best model to reduce falses positives.

Allele frequency affects the power of GWAS analysis. Rare alleles (present in a few samples) are ignored since most GWAS analyses remove minor allele frequency MAF present in less than 5% of germplasm.

Linkage mapping explores rare variants than GWAS. Other approaches that exploit both linkage and GWAS, such as Nested Association Mapping NAM population, reduce population structure and falses positives observed with natural populations. Multiparent Advanced Generation Inter-Cross (MAGIC) population increases allele diversity in the mapping population [4,33].

Determining linkage disequilibrium LD before the GWAS study is essential since it allows for delimiting the QTL interval and defining significant SNP. LD determines the interval for candidate gene search and indicates the number of markers to saturate a genome scan and mapping resolution. LD in the population reflected a non-random association of alleles at different loci. The coinherited SNP shows a high r^2, mainly representing the same QTL on the same chromosome and vice versa. LD can be displayed in scatter or heatmap plots as r^2 versus genetic or physical distance. Genome size, population type, and mating mode are among the factors affecting LD. For instance, self-pollinated crops like barley have a large LD decay. They require fewer markers because homozygotes can be easily at any loci than outcrossing crops like maize [4,51].

4.2. Interpretation of GWAS results

The Manhattan and quantile-quantile (QQ) plots are the main plots used to report the GWAS result. The Manhattan plot shows on the x-axis SNP position along the seven barley chromosome, and the Y-axis shows ($-\log_{10}$ p-value) of the associated SNP. A dot represents each SNP. The more significance corresponds to the small p-value and the high $-(\log_{10}$ p-value). $-\log_{10}$p equals three is mainly used as a threshold for significant markers. The QQ plot shows on the X-axis's expected p-value and the Y-axis's observed p-value. We can infer from the plot if the population structure is controlled [4,51]. If the graph's start shows inflation, the p-value deviated from the diagonal line, then population structure was not controlled well or indicates a measurement bias; if they are well aligned, it shows a good calibrated study.

In order to further apply GWAS results in plant breeding, GWAS is followed mainly with an understanding mechanism, function, and activity underlying the QTL. Identifying which cell and tissue are involved, the causal nucleotide, determine other factors in which the protein is expressed, for instance, in the presence of a pathogen or drought, which allele increases or decreases the trait. Breeders select the desired trait depending on the desired direction for the trait [4,38].

GWAS results can guide breeding. For instance, searching for candidate genes can be followed by gene editing. They can also be applied in breeding via MAS or genome selection. However, it is essential to validate results using both approaches, GWAS or linkage mapping, and use different materials [4,52]. Resources to interpret GWAS results and gain insights into the mechanism underlying each QTL or MTA include databases and servers to annotate the identified QTL or MTA. Ensembl, Barley map, BarleyVarDB, BARLEX, and GrainGenes are among the databases containing the barley genome sequence and other information used to interpret GWAS results [38].

5. Main barley breeding objectives

Yield increase and stability are the top goals for barley breeding. Yield component traits such as thousand-grain weight, grain per spike, days to maturity, flowering time, plant height, and grain number per spike are essential traits in barley breeding and are highly associated with yield. Genes and QTL associated with these traits have been identified and are being used in barley breeding [4]. For instance, semi-dwarf cultivars are associated with high yield due to less lodging. In barley, the semi-dwarf genes mainly used are (*uzu1*) semi-brachytic 1, breviaristatum-e (*ari-e*), and (*sdw1*) semi-dwarf 1 [53]. Thousand-grain weight (TGW) shows the average weight of grains; the increase in the number of grains per spike increases the overall yield. Awns intercept light and thus play a key role in photosynthesis [54].

However, disease and biotic stress reduce yield and grain quality, especially fungi *Blumeria graminis f. sp. hordei, the* causal agent of powdery mildew (PM), and *Pyrenophora teres f. teres*, the causal agent of the net form of net blotch (NFNB). Yield loss is estimated to be up to 30% and 40% for PM and NFNB in

favorable conditions of the pathogens, respectively [55,56]. Thus breeding for disease resistance remains a priority for barley breeders.

Quality breeding for barley depends on the end uses; Grain size and protein content are important traits for malting barley. In addition, β glucan and micronutrient content such as Zn and Fe in barley are essential traits for human consumption. Increasing micronutrient contents in staple crops will help alleviate malnutrition, especially in rural areas. Iron and zinc deficiencies affect more than 50% and 30% population worldwide, respectively [57].

Additionally, climate change has impacted crop production, therefore, food security by elevating principal abiotic stress related to soil quality (salinity, nutrient deficiency, fertilizers, toxicity, PH-acidity), water stress (drought, flooding), and temperature stress (heat and cold stress) [2]. All these abiotic stress cause yield losses estimated up to 70%, reduce grain quality, and thus economic loss, especially for malt barley [58]. The loss depends on the plant growth stage, the length of the stress, soil quality, and cultural practices. For example, in the case of heat stress plant time, earl planting and shorter seasons reduce its effects [14].

On the other hand, water deficiency (drought) remains the limiting factor for crop performance and causes more yield reduction. Therefore, breeding for abiotic stress tolerance cultivars remains a high priority in many breeding programs. Exploiting wild relatives of barley, the variation found in cultivated barley, especially landraces will offer new alleles and resistance sources for abiotic stress tolerance. For instance, Saade et al [59] showed that wild alleles on 2H from *H. spontaneum* increase yield in salt conditions.

5.1. Breeding for disease resistance

Breeding for disease resistance is the second target trait after yield. Managing diseases is essential in achieving food security. Barley is attached by many living organisms (biotic stresses), such as fungi, bacteria, viruses, insects, and pests. They attack different parts of the plant: ear, leaf, leaf sheaths, stem, and grain. They reduce yield and grain quality and cause economic loss [37]. Some pathogens are more relevant in some regions than others depending on climates conditions (temperature, light, humidity, water**)**, planting time, genetics of the pathogen (the inoculum quantity, its survival on no host plant), and soil quality [60]. On the other hand, climate change has increased incidence and disease severity [61].

Plant disease management includes cultural practices, pesticides, and resistant cultivars. Cultural measures such as removing debris, rotating crops, and adequate soil quality reduce disease incidence but are less effective. Chemical uses, including pesticides, and fungicides applied on seeds or plants, are most effective. However, they show several adverse effects such as recurrent expense, development of resistance in pathogens accompanied by the emergence of new races in pathogens, and toxicity for living creatures and the environment. Thus, pesticide regulations emerged in some countries, mainly from the Europe Union [62,63].

Breeding resistant cultivars are the best way to manage disease long-term since they are low cost for poor farmers with no means to buy pesticides. They are environmentally friendly because they limit the use of pesticides. It requires screening and selecting for resistance genes in germplasm diversity, transfer or introgressing the gene into an adapted line. The insertion or incorporation technics will depend on the gene pool of the source of resistance. Suppose resistance genes are in the primary or second gene pool. In that case, technics such as crossing, recurrent selection, or marker-assisted backcross (MAB) will be used. On the other hand, if the resistant gene is from distant wild species, genetic transformation techniques will be used [64].

Modern tools for breeding for disease resistance (gene editing, QTL mapping, genome selection, genetic engineering) are faster and more effective than classical breeding for disease resistance [37]. Recently genome editing tools such as CRISPR/Cas9 can offer precise mutation and are considered no genetically modified organisms (GMO-free) in some regions. Kis et al [65] used CRISPR/Cas9 to develop barley lines resistant to the *wheat dwarf virus.*

Resistance types in plants can be qualitative or quantitative. Qualitative resistance, when major resistance R genes offer resistance, is often race-specific and often breaks down due to pathogen evolution. On the other hand, quantitative resistance is when many genes offer resistance and is often more durable than qualitative [66].

Among barley pathogens, viruses and fungi are the most damaging. The most damaging viruses of barley are the barley yellow dwarf virus (BYDV) caused by the aphid, the barley mild mosaic virus BaMMV, and barley yellow mosaic virus BaYMV, which are soilborne [67]. Fungi diseases caused a 15% yield loss. Powdery mildew and NFNB are among the relevant fungi pathogen, causing considerable economic loss [68].

5.1.1. Net form of net blotch (NFNB)

Two forms exist of net blotch, the net form caused by *Pyrenophora teres f. teres Ptt* and the spot form caused by *P. teres* f. *maculata Ptm.* Both forms are difficult to differentiate morphologically but can be done genetically with PCR primers. They appear on leaves, leaf sheaths, and glumes. *P. teres f. maculata (Ptm)* shows circular lesions*;* it starts as a biotroph and then continues to be a necrotroph lifestyle; it is called hemibiotroph. *Pyrenophorateres f. teres Ptt* shows longitudinals, narrow, brown chocolate net-type striations. It lives in dead cells. Therefore, it is a necrotroph. It can survive in barley debris, which constitutes an inoculum that infects new plants. Contaminated seeds and wild grass are also a source of inoculum. *Ptt* toxins quantity affects the severity of the symptoms [69,70]. *Ptt* penetrates the leaf with oval lesions on the infection area within 24h of infection. It extends along the leaf vein in striations of net type; the striations are surrounded by chlorosis [71,72]. In favorable conditions for *Pyrenophora teres f. teres* development of humidity above 75% and temperature between 15 and 23°C; yield loss is estimated to be 10 to 40% [37]. It reduces grain size, malt, and overall grain quality [69].

Taxonomy: *Pyrenophora teres* Drechs belongs to the Kingdom Fungi; Phylum Ascomycota; Subphylum Pezizomycotina; Class Dothideomycete; Order Pleosporales; Family of Pleosporaceae; genus *Pyrenophora*, form *teres*, and form *maculata, species: Pyrenophora teres [69]*.

Barley harbors several NFNB resistances loci. Major resistance genes and QTL have been identified on all barley chromosomes.

Rpt2 on chromosome 1H; *Rpt3, Pt.d* on 2H; *Rpt1,Pt.a* on 3H; *Rpt7* and *Rpt8* on 4H; *Rpt6* on 5H; *Rpt5, rpt.k, rpt.r* on 6H; and *Rpt4* on 7H. Chromosome 6H harbors many loci for NFNB resistance [73,74]. Moreover, several studies applied QTL mapping to NFNB in barley [75-77].

5.1.2. Powdery mildew

Powdery mildew on barley is caused by *Blumeria graminis f. sp. hordei*. The symptoms are gray to white powder on leaf and leaf sheaths. In favorable conditions, with high humidity above 85% and temperature 12^0C to 20^0C, yield loss of about 40% is observed [78]. It is a biotroph since it grows and reproduces in live host cells. When it infects the plants, it penetrates the epiderm cell membrane. Once inside, it forms haustoria, a feeding structure for nutrient uptake [79].

Taxonomy: *Blumeria graminis f. sp. hordei* belongs to the Fungi Kingdom, Phylum Ascomycota, Subphylum of Pezizomycotina, Class Leotiomycetes, Order Erysiphales, Family Erysiphaceae, Genus Blumeria, and specie of *Blumeria graminis f. sp. hordei* [80]**.**

Barley resistance to PM relies on two primary loci, *Mla* (Mildew Locus A), on 1H. Mla loci include many alleles and are race specific. The second is the *mlo (*mildew resistance locus o) on 4H. *Mlo* offers broad-spectrum resistance; it originated from Ethiopian landraces and has been used for *Bgh* resistance for a long time. However, it favors the cultivars' susceptibility to necrotrophic and hemibiotrophic pathogens. *Mlo* is the race no specific loci and offer durable resistance. Other PM loci have been identified and are currently being utilized for PM resistance: *Mlat, MlGa, Mlk, Mlnn, Mlra, Ror1, and Mli* on 1H; *MILa, MlHb, and MlMor* on 2H; *Mlg, MlBo on* 4H; *Mlj* and *MlTr on* 5H; *Mlh on* 6H; *Mlf* and *Mlt on* 7H [73,81]. Resistance genes can break easily, thus the necessity of developing cultivars with a broader resistance spectrum. Genomic loci associated with PM in barley have been analyzed via QTL mapping [81,82]. [83] mapped two QTLs on 7H that show 45% of phenotypic variation.

Sexual reproduction and mutation lead to many races found in *Ptt* and *Bgh.* They overturned the resistance mechanism of barley cultivars and fungicides, making breeding for durable resistance a challenging task. Thus, the need to find new sources of resistance to these pathogens is required. Gene pyramiding of major resistance genes into elite cultivars will help achieve durable resistance. Breeders need to exploit and harness the genetic diversity found in all barley gene pools, especially landraces and wild, since they are sources of resistance to disease [60,70].

5.2. Breeding for micronutrients increase.

Yield for staples crop has been the target trait primarily due to the increase in the world population. While quality, such as micronutrients, was not on the radar of breeders. Two-thirds of the worldwide population is undernourished (lacking at least one vitamin and mineral). 60% of the world population has iron deficiency, and 30% lacks zinc [57]. Malnutrition or hidden hunger impact developing countries and affect primarily women and children. It is a public health crisis. Thus, food production should be paired with nutritional value, especially micronutrients.

Micronutrients participate in several vital functions in the human body, such as coenzymes and the biosynthesis of hormones [84,85]. In the body, iron is found in hemoglobin; it assures oxygen transport. On the other hand, zinc is involved in many enzymes and immune and nervous systems. Fatty acids (FA) in the

body are in the cell membrane, stores carbon and energy, hormone synthesis, and many more [86]. Symptoms in children are poor growth, stunted growth, blindness, low intelligence (IQ), slow intellectual development, poor immunity, and death. Fe deficiency is the leading cause of anemia and impaired growth in children. Zinc deficiency is responsible for stunted growth [85].

5.2.1. Genetic approaches to biofortification

Genetic biofortification requires modification at the genetic level of the plant. It is effective long term, increases micronutrients, and can reduce antinutrients. Genomics approaches for micronutrient breeding include using biotechnology tools to explore pathways and mechanisms associated with micronutrient uptake, transport, and storage into edible parts of the crop [87]. Conventional breeding approaches rely on available genetic variation in the gene pool; focus on crossing genotypes with high micronutrients. It uses marker-assisted selection (MAS) tools, to select lines with high microelements content. Moreover, QTL mapping is used to select MTA with micronutrient content, which can be used in MAS. Conventional breeding is more accepted than using genetic engineering techniques [88,89].

Genetic engineering is not limited to the available diversity. It is more effective and faster than conventional breeding. Tools such as gene transfer, gene editing, and transgene insertion, which increase microelements content, achieved high results. However, their acceptance and regulations are still challenging—for instance, the acceptance of golden rice developed by genetic transformations. Golden rice is a high micronutrient and beta (β) carotene-rich rice variety, resulting from selecting three enzymes [90,91].

Mutation breeding creates variation that can be exploited to breed high micronutrients contents lines. It can lead to a new and improved trait. However, mutations by radiation and chemical are often uncontrolled. Genome editing has increased the precise mutation. It uses programmable nuclease such as zinc finger nucleases (ZNF), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats and associated protein Cas CRISPR/Cas. CRISPR/Cas9 has become widely popular for gene editing. For example, CRISPR was used to mute a grain phytase gene *HvPaPhy a*, which degrades phytic acid [92]. It increased FA in rice by knocking out the *FAD2*, fatty acid desaturase 2 [93].

5.2.2. Genes associated with Zn and Fe accumulation

Several genes and proteins are associated with Zn and Fe accumulation in grains from soil uptake, transport, and loading in grain. Transport and chelators of both Zn and Fe overlap; improving one leads to improving the other. For instance, Nicotianamine Na intervenes in the transport and mobilization of both elements [87]. NAS synthase synthesizes NA. Overexpression of NAS of rice *OsNAS2* and FERRITIN (*Pv FERRITIN*) that stock Fe of the bean into wheat resulted in high ZN and iron in grains [37]. Moreover, The iron-regulated transporter 1(*HvIRT1)* from the ZIP family transport Fe and Zinc [89]. Overexpressing the chelators and transport proteins of Zn and iron have increased Fe and Zn content [86].

In low Fe conditions, cereals release phytosiderophores via roots into soils. The phytosiderophores play a crucial role in the solubilization and chelators of Fe and Zn. Another element chelator is NA (nicotiamine), an intermediate precursor of phytosiderophores [87]. Transgenic rice expressing nicotianamine aminotransferase (NAAT) of barley secreted more phytosiderophore than control and showed a high level of Fe [94]. Nicotianamine aminotransferase enzymes (*HvNAAT*), deoxymugineic acid synthase (*HvDMAS)* participate in Fe uptake. Two enzymes NAAT (nicotianamine aminotransferase) and NA (Nicotianamine) synthase, regulate nicotianamine (NA) levels in plants. For example, in transgenic rice with the barley gene (*HvNAS1*), a NA synthase showed a high level of Fe content in seeds [95].

Yellow stripe-like proteins (*YSL)* carry *Fe*. Ferritin proteins store Fe for later usage and liberate it when needed under low Fe conditions. It prevents Fe oxidation. Thus, expressing storage protein, such as ferritin, increases Fe in grains. VACUOLAR IRON TRANSPORTER (*VIT*) assures the loading of Fe in grains [89].

The main proteins that assure zinc and Fe transport in barley include Zinc induced facilitators like Transporter ZIFL4, transporter of mugineic acid family *TOM1*. ZIP transporters (*HvZIP*) assure Zn soil uptake, translocation, and loading into grains. Another HEAVY METAL TRANSPORTING ATPase, *HMA2*, and *HMA4* [89].

Mapping of QTL associated with Zn and Fe is essential to identify new alleles and genetic loci to understand their function, mechanism, and expression of the associated candidate gene. The associated markers can be used in breeding, e.g., MAS, to develop higher Zn and Fe cultivars. Studies by [96-98] have used QTL mapping in large barley germplasm to identify genomic loci associated with Fe and zinc and reported

higher Fe and Zn content. [99] mapped QTL associated with Zn remobilization into barley grains in 150 mapping populations from cultivar Sahara and Clipper. The cultivar Sahara alleles were associated with high Zn remobilization into grains (37%).

6. Conclusion

Barley (*Hordeum vulgare L. ssp. Vulgare*) is the fourth most important cereal worldwide and the second in Morocco. It constitutes a staple crop in several regions, including North Africa. Moreover, it contributes to the economic increase mostly due to malt. Therefore, increasing yield production is essential. However, diseases such as NFNB and PM reduce yield and grain quality, making breeding for disease resistance an essential goal in barley breeding. On the other hand, breeding cultivars with higher zinc and iron will alleviate malnutrition, which constitutes a public health crisis, especially in developing countries.

Genetic diversity results can be used in breeding programs and conservation. For instance, to select parents that can be used in crosses.

On the other hand, GWAS has become popular in discovering Quantitative trait loci QTL and Marker trait association MTA associated with important traits in agriculture. It is having been preferred over linkage mapping since it fast, and more alleles can be analyzed at once. Associated markers identified for traits such as disease resistance, zinc, and iron content in grains represent rich information for barley breeding via MAS and genome selection. Once they have been validated in other populations or confirmed in other experiments. Once validated, they can be used in barley breeding; candidate genes can be cloned, and technics such as gene editing targeting the candidate genes can be considered.

Conflict of Interest

The authors declare that they have no conflict of interest.

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