Identification of genetic basis of agronomic traits in alfalfa (*Medicago sativa* subsp*. sativa)* using Genome Wide Association Studies

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Article info	Abstract
  OPEN ACCESS ***Corresponding author** mounawer.badri@cbbc.rnrt.tn **Conflict of Interest:** The authors declare no conflict of interest**.**	Alfalfa (*Medicago sativa* L.) is one of the most widely cultivated forage crops due to its high yield, value of nutrients, and adaptation to diverse environments. However, many of the desired agronomic traits in alfalfa are quantitative, and therefore improving them under abiotic and biotic stresses becomes an important goal in *alfalfa* breeding. One way to achieve such a goal is identification of loci that contribute to variation in complex traits. Genome Wide Association Studies (GWAS) provide advanced tools to identify genetic loci associated with traits of interest using high-density markers throughout the genome. In *M. sativa*, previous studies indicated that GWAS could identify numerous candidate single nucleotide polymorphism (SNP) markers for quantitative traits, including biomass, yield, forage quality, and drought/salt tolerance. Furthermore, mapping SNP markers against the *M. sativa* reference genome revealed many putative candidate genes which are associated with several cell wall-related traits. In this review, we summarize Genome Wide Association (GWAS) in alfalfa from concept to application and the identification and characterization of candidate genes for traits of interest.

1. INTRODUCTION

Alfalfa is selected from a complex taxonomic group called *Medicago sativa* species complex, that belongs to *Fabaceae*, and it is an allogamous perennial legume. Alfalfa is one of the earliest domesticated and the most valuable forage legume crops that plays a significant economic role worldwide (Azzam et al., 2019) and the most cultivated forage legume in Mediterranean-climate regions (Annicchiarico et al., 2015). The *Medicago sativa* species complex includes both diploid (2n = 2x = 16, with a small genome size of 550 Mbp) and tetraploid (2n = 2x = 32, with a genome size of 800–1,000 Mbp) subspecies (Yu, 2017; Sakiroglu and Mavioglu Kaya, 2013; Sakiroglu, 2021). Alfalfa has been widely used for animal feed for its high nutritive value, with rich levels of nutrients that include high quality protein and amino acids, dietary fiber, vitamins and bioactive molecules (Bhattarai et al., 2020). It also has important benefits for sustainable agriculture systems because of its deep root system and perennial nature that limits top soil erosion. Alfalfa exhibits a relatively high level of tolerance to drought and salt stress (Badri et al., 2021; Jabri et al., 2021). Consequently, different studies on alfalfa improvement have led to the release of cultivars with superior performance such as high yield, better stress tolerance or forage quality. Identifying the genetic basis determining intraspecific variations in various agronomic traits, which may provide resources and information for alfalfa breeding, is a challenge for improving alfalfa productivity under environmental constraints (Zang et al., 2015; Yu et al., 2017; Sakiroglu and Kang, 2022). Food and feed production, and therefore food security, face several threats as a result of vulnerability to climate change that gives rise to

numerous environmental stresses, including soil salinity (Raza et al., 2022). Salt stress is a major abiotic factor affecting global agricultural productivity by disrupting numerous physiological, biochemical, and molecular processes in plants. In particular, salinity affects alfalfa growth, development, yield and nutritive quality. In Tunisia, 9.13% of the total surface of the country and approximately 25% of the cultivated areas have been affected by salinity (Arraouadi et al., 2011). However, improving salt tolerance in alfalfa to maintain its sustainable productivity has become a challenge.

Therefore, to fulfil the global food demand under stress conditions, a wide range of plant varieties with a improved performance in terms of yiled stability is needed. Various traits of importance for this stability include yield-related and stress-inducible responses, which are controlled by numerous genetic loci and significantly influenced by the environmental conditions as well as genotype_environment interactions (Li and Brummer, 2012). To capture the desired performance in such quantitative traits in many crops, different mapping techniques have been used to map the quantitative trait loci (QTL) and incorporate them into breeding programs to accelerate breeding efforts. Therefore, advances in genomic technologies and the examination of trait variation across diverse genetic backgrounds were the major driving forces behind the initial wave of mapping studies (Zhang et al., 2020). Recently, Next Generation Sequencing (NGS) technology has been used for developing high density markers (single nucleotide polymorphism, SNPs) at the genome-wide scale across many accessions/individuals/genotypes (Hawkins and Yu, 2018). QTL analysis has been a highly significant and efficient mapping approach that utilizes a wide range of molecular markers (more recently SNPs) by producing marker-trait associations that are intended for use in downstream selection of the complex traits (Nakano and Kobayashi, 2020). Linkage disequilibrium (LD) mapping, also known as association mapping, demonstrates the statistical relationship between the genetic markers and phenotypes within natural populations, which constitutes an effective and highly precise approach for testing association between genome-wide polymorphisms and phenotypic traits (Kondratyev et al., 2021). Genome-wide association study (GWAS) is a powerful approach for partitioning the genomic regions involved in target trait based on association analysis between genome-wide SNPs and phenotypic variation with higher resolution compared to the biparental mapping approach (Larkin et al., 2019; Porter and O'Reilly, 2017). Previously, GWAS have been exploited in alfalfa to identify markers associated with drought tolerance (Zang et al., 2015), salt tolerance (Yu et al., 2016), forage yield (Li et al., 2011; Şakiroğlu et al., 2012; Şakiroğlu and Brummer, 2017) and forage quality (Li et al., 2011; Şakiroğlu et al., 2012; Şakiroğlu and Brummer, 2017; Biazzi et al., 2020) and *verticillium wilt* resistance (Yu et al., 2017).

2. GENOME WIDE ASSOCIATION STUDIES: FROM CONCEPT TO APPLICATION

2.1. Genetic resolution

Understanding the genetic structure of complex traits is essential to comprehension of plant biology. Most traits of agricultural value are complex traits that are governed by several genetic loci and are under environmental influence (Tibbs et al., 2021). Progress in genomic and computational technologies, methodology development, and the desire to study trait variation throughout various genetic backgrounds were the main drivers behind the first wave of association mapping studies in model plants and crop species (Morris et al., 2013).Moreover, focus to identify the genetic control of phenotypes among many individuals in a wide range of environments is of major fundamental interest. In fact, Burghardt et al. (2017) explained the use of two major approaches to study genotype/trait associations using mapping populations which are QTL mapping, and linkage disequilibrium (LD) mapping. Recently association mapping has been developed based on candidate gene association mapping and genome wide association study (GWAS) (Zhu et al., 2021).

GWAS is an observational study of a genome-wide set and it is an efficient tool for linking the genotype–phenotype in natural populations to detect association between allele or genotype frequency and trait status (Li, 2020). Additionally, GWAS has numerous advantages over biparental QTL mapping (Table 2). It provides a higher resolution for localizing a QTL and overcoming the limitations of biparental mapping (Ma et al., 2022). Through screening large and diverse collections with ample genetic marker density, GWAS can precisely detect polymorphisms and the underlying genetic loci that are responsible for phenotypic variation under biotic and abiotic constraints (Afzal et al.,

2022). GWAS uses the existing LD to detect genetic variation associated with the phenotypic variation for the traits (Xiao et al., 2017). Several studies proved that the resolution of GWAS depends on LD extent, which is usually related to the species mating system (Bilton et al., 2018). Therefore, Huang et al. (2012) estimated that the resolution of the GWAS mapping in lines of the wild outcrossing species *Oryza rufipogon*, for which LD decays rapidly over ~20 kb, is three times better than that in the autogamous cultivated subspecies *Oryza sativa* for which LD decays over ~150 kb, using the same number of markers.

GWAS have been successfully applied to detect the causative allele(s)/loci which can be used in

Table 1. The advantages and limitations of GWAS mapping

Advantages	Limitations
No parents or crossing is needed	Population structure
highest number of contrasting accessions	Not efficient with the lower number of individuals
Can encompass high allele richness	Requires a large number of alleles across the genome
Wide phenotypic variation	Elevated false positive associations
Higher at marker positions	High heritability value is required

the breeding of crop plants for adaptation and yield improvement. In GWAS, the genotypic data consist of genome-wide single nucleotide polymorphisms (SNPs) detected through array-based genotyping, genotyping-by-sequencing or resequencing (Rafalski, 2010). The use of SNPs in GWAS has a particular importance to examine genetic variations as SNPs are the most common and easily identifiable genetic variation across the genome. SNPs are less informative than SSRs because they are predominantly biallelic in nature. The expected heterozygosity is also lower for SNPs than for SSRs, leading to successful assessment of population structure and family relationships in most crops (Wessinger et al., 2018). Thousands to millions of SNP loci from hundreds of individuals/accessions/genotypes can be genotyped using high throughput markers owing to recent breakthroughs in genome wide

genotyping technologies (Naveed et al., 2018; Tondelli et al., 2013).

The genetic resolution of GWAS depends on the availability of a set of polymorphisms that are densely dispersed across the genome (e.g. SNP) (Ogura and Busch, 2015). However, the power of GWAS also depends to the heritability of the trait and availability of the phenotypic variation in the mapping panel. On the other hand, the presence of rare allelic variants with large effects increases false positives (Asimit and Zeggini, 2010) in addition to the hidden population structure in the mapping panel. This is due to the fact that some individuals are more closely related within the population (Zhu and Yu, 2009). Thus, large population size, high number of markers, and accurate phenotyping over several environments are required to effectively perform GWAS (Manchia et al., 2013) as the resolution of GWAS gradually decreases with an increase in the number of undetected SNPs responsible for the phenotype (Visscher et al., 2017)**.**

2.2. Phenotyping challenges

Plant phenotype includes all agronomic, morphological, physiological and biochemical traits that reflect plant structure, growth and composition (Houle et al., 2010). This includes not only agronomic traits such as structure, size and color, but also physiological states during development. Understanding the phenotypic variation in plants is key to the identification of the biological determinants and how a plant's genetic background interacts with its environment (San-Cristobal et al., 2022). Therefore, phenotypes determined by a particular genotype under an environmental condition are specific to that environmental condition and can change with subsequent environmental changes. This type of change is deemed as a key for survival and plant productivity in a variety of natural environments (Mitchell-Olds, 2010).

As previously mentioned, GWAS reveal associations between phenotypes and SNP markers based on historical LD. However, it is difficult to identify all QTLs or SNPs in close vicinity of the QTLs in GWAS (Xiao et al., 2022). GWAS approaches have been used to detect marker loci that are associated with traits in many crops but certain challenges persist. One such challenge is phenotyping (Feng et al., 2017). Therefore, measuring and data handling for the phenotypic variation is a challenge to GWAS for complex traits (Table 1). In fact,

phenotypic data should be evaluated, filtered from outliers prior to analyses. In addition to the outliers, deviations from normal distribution poses a challenge for GWAS analyses. Moreover, heritability estimates are a good indicator of how much genetic variance contributes to a phenotype and how closely a phenotype is related to the relevant genotypes. Only traits with moderate to high values (for filtered phenotypic data) should be included in the GWAS. Low overall heritability is a limiting factor that reduces the ability of GWAS to recognize associations (Fung and Luo, 2018). As genotype x environment interaction is a factor in low heritability, it should be assessed. In addition, GWAS performed with small population sizes reduces the power of tests, and therefore, detection of loci-trait associations. Hence, increasing the population size will improve the power of GWAS tests. Population structure presents yet another challenge but can be controlled by statistical approaches. Various methods of relatedness can be empirically estimated from genotypic data and be incorporated into analyses to reduce false positives. For instance, Bayesian clustering algorithms implemented in the software program STRUCTURE (Prichard et al, 2000) and principal component analyses (PCA) as well as pairwise kinship matrices have been used to delineate the hidden population structure and subsequently incorporated into analyses in a mixed-model approach (Martins et al., 2022) to reduce the false positives arising from the family structure. Neglecting corrections for population structure leads to an elevated number of false associations between genotypes and traits of interest (Ibrahim et al., 2020).

2.3. Interpretation and use of genetic and phenotypic association data

The basic approach for conducting GWAS in crops has been illustrated by several researchers (Alseekh et al., 2021; Kondratyev et al., 2021) in the following steps (Fig. 1): (i) Select a panel of accessions/individuals or genotypes to form the mapping panel, (ii) collect phenotypic data of all the individuals/accessions under replicated field trials preferably from those that have a moderate to high value of heritability, (iii) collect genotypic data from the individuals or accessions (preferably SNP markers), (iv) determine the population structure within the mapping panel, (v) carry out the statistical analyses that associate the genomic variants to phenotypic variation. Generally, the mixed linear
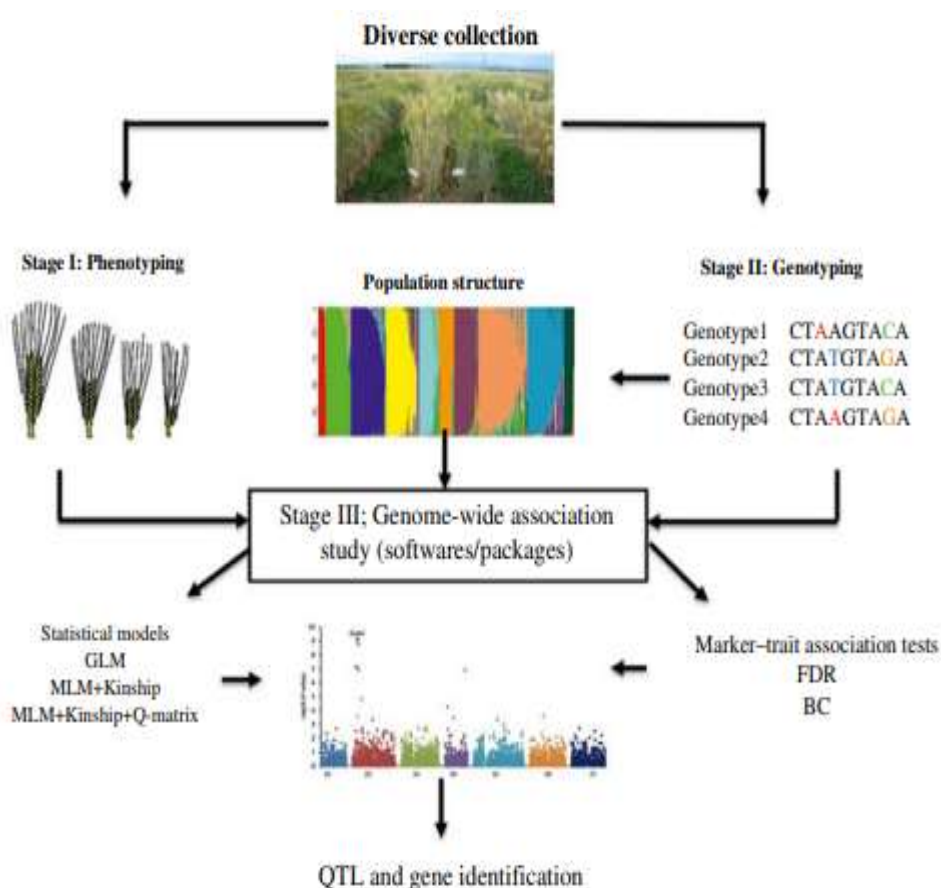

Fig. 1. Methodology to conduct GWAS in crop plants (from Alquadah et al., 2020).

models or that also consider the population structure are proposed for performing GWAS, (vi) interpret the results of the analyses to determinate a set of SNPs that are candidates responsible for traits of interest, (vii) prioritizing candidates for follow-up work is usually the following step for GWAS analyses that includes evaluation of the strength of statistical association, complementary mapping data, biological knowledge, genomic annotation, and expression data, and (viii) validate candidate genes throughout an independent studies.

The GWAS approach has been proven successful in various economically valuable crop plants for the identification of candidate loci controlling important traits such as flowering process, resistance to rice yellow mottle virus and fertility restoration in rice (Yano et al., 2016; Cubry et al., 2020); compositional and pasting properties (Alves et al., 2019), stalk biomass and leaf cuticular conductance (Lin et al., 2020) in maize; grain size and grain quality (Kimani et al., 2020) in sorghum; lodging, productivity in beans (Resende et al. 2018); and cotton architecture (Su et al. 2018). In addition, the stress tolerance and the seed quality traits in brassica (Lu et al., 2019); the yield and drought tolerance related traits in sesame (Li et al., 2018) were performed by GWAS.

3. GWAS IN ALFALFA: VALIDATION AND APPLICATIONS FOR AGRONOMIC TRAITS

3.1. Agronomic traits and molecular markers in alfalfa

M. sativa is one of the most popular *Medicago* species in the world today because of its many outstanding characteristics such as salt tolerance, cold resistance, wide adaptability, high yield, nutritive quality, resistance to a wide array of diseases, and being widely planted throughout the world (Kumar, 2011; Li and Brummer, 2012; Wang et al., 2018).

The nutritive value of alfalfa and its forage characteristics can be assessed by several key parameters, such as protein concentration, total fiber concentration and the types of fiber present. It depends mostly on the forage digestibility, and leaf-to-stem ratio which are determined by the acid detergent fiber (ADF) and acid detergent lignin (ADL) concentrations (Lin et al., 2020). The other agronomic trait controlling alfalfa intake is the concentration of neutral detergent fiber (NDF) (Biazzi et al., 2017). Biazzi et al. (2019) demonstrated that improving fiber digestibility, protein quality and decreasing the rate of protein degradation in rumen are the major breeding goals towards alfalfa quality improvement.

Alfalfa yield production and nutritive value have been limited by numerous abiotic (drought, high salinity and nutritional deficiencies) and biotic (fungi and insects) constraints. Drought resistance is an important trait for improving alfalfa productivity under water deficit conditions (Wang et al., 2020). In addition, winter hardiness is another important agronomic trait for alfalfa that affects biomass yield and complicates alfalfa improvement (Robins et al., 2007). Efforts toward the developing alfalfa cultivars resistant to *Verticilium* have been released using a traditional breeding strategy (Yu et al., 2017). Since a major part of desired agronomic traits are under the control of several genes and interact with environmental factors, development of new alfalfa cultivars with high regional adaptation and persistence is an important goal for breeding selection throughout the identification of loci that contribute to variation in such complex traits (Annicchiarico, 2021).

Various criteria such as morphological, agronomic and physiological traits, pedigree records, molecular markers, or combinations of criteria are used to improve alfalfa (Li and Brummer, 2012). Methods that incorporate molecular/genomic markers have many advantages over traditional phenotype based selection methods because molecular/ genomic markers are readily detectable and stable in plant tissues without environmental influences (Jia et al. 2017; Li and Brummer, 2012). In fact, the use of molecular markers in alfalfa started with the discovery of early molecular marker techniques and evolved concomitantly. Restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPDs) (Nagl et al., 2011), amplified fragment length polymorphism (AFLP) (Segovia-Lerma et al., 2003), DNA amplification fingerprinting (DAF), sequence characterized amplified regions (SCARs), microsatellites (Simple Sequence Repeat) (SSR) (Flajoulot et al., 2005; Bagavathiannan et al., 2010; Şakiroğlu et al., 2010) have been used in alfalfa genetic diversity. Al-Farsi et al. (2020) evaluated the genetic diversity of 34 alfalfa accessions/landraces from Oman, which clustered together into seven groups. Azzam et al. (2019) reported a highly salt-tolerant alfalfa population by screening 16 alfalfa populations using inter simple sequence repeat and RAPD markers. Jiang et al. (2015)

have used RAPD markers to cluster 25 salt-tolerant alfalfa populations into 9 clusters. They suggested that alfalfa germplasm have a high genetic diversity which can be involved in salt-tolerant breeding.

Recently, the genotyping-by-sequencing (GBS) method provides a cost-effective method for genotyping high-density SNP markers in alfalfa that could be utilized in downstream analyses of genetic diversity or GWAS (Şakiroğlu et al 2017; Munjal et al., 2018), which can be applied to analysis of the genetic diversity as well as facilitate the identification of SNP markers associating with phenotypic traits (Hawkins and Yu, 2018) or environmental predictors. Quantitative trait locus (QTL) mapping has been used for mapping genetic loci associated with several alfalfa agronomic traits such as yield, plant height (Robins et al., 2007) for winter hardiness, fall dormancy (Adhikari et al., 2018), plant growth, forage quality traits and stem histology (Inostroza et al., 2021). Pierre et al. (2011) used a fine mapping method to find a major flowering time QTL on chromosome 7. In addition, Ray et al. (2015) reported a small-effect QTLs controlling biomass yield trait under drought in tetraploid alfalfa. Similarly, Li et al. (2015) found 71 QTL associated with plant height and winter injury, and these QTL were linked to yield of alfalfa in an alfalfa F1 population. Zhang et al., (2020) identified Twenty-eight QTLs associated with flowering time and they suggest that the identified markers could be used for marker-assisted selection in breeding programs of alfalfa varieties. However, despite some advantages, biparental QTL mapping approach has some limitations such as laborious, time-consuming mapping panel formation and low resolution of the QTL. Genome-wide association studies (GWAS) are a powerful strategy that can overcome the limitations of conventional QTL mapping.

3.2. Overview of GWAS in *M. sativa* and its limitation

Several morpho-physiological and biochemical traits that contribute to forage quality are important breeding goals. Using GWAS to identify molecular markers closely associated with genomic regions that control these traits may improve forage and feed quality (Sakiroglu and Brummer, 2017). In alfalfa, many functional loci associated with salt stress tolerance, drought stress tolerance, forage quality, and disease resistance have been identified via GWASs (Yu et al., 2016; Jia et al., 2017; Biazzi et al., 2019).

In recent years, a number of association mapping studies using GWAS have also been conducted. Sakiroglu et al., (2012) used 89 SSR markers from 120 accessions and phenotyped individuals for cell wall constituents and biomass yield related traits. A mixed linear model was used to test associations and three significant markers were found. Zhang et al. (2015) performed a GWAS approach on 198 accessions of *M. sativa* for drought resistance. They identified 26,163 SNP markers with 19 of these showed a significant association with target agronomic traits. Yu et al. (2016) genotyped 198 *M. sativa* accessions, phenotypes for germination rate under salt stress and performance of GWAS 4,653 SNP. Of these, 23 SNPs showed a significant association with salt tolerance during germination. The same panel of accessions for mapping loci associated with four agronomic and physiological traits (dry weight, plant height, leaf chlorophyll content, and stomatal conductance) under salt stress was genotyped by throughout GWAS (Liu and Yu, 2017). This study identified 42 SNPs significantly associated with salt tolerance. Similarly, Yu et al. (2017) used a panel of 179 breeding lines which was phenotyped for *Verticillium wilt* tolerance. The GWAS analysis using 19,801 SNP identified 10 SNPs significantly associated with *Verticillium wilt* resistance. A total of 374 individual genotypes from 120 accessions of *M. sativa* were phenotyped for 23 yield and nutritive value related traits. The traits were evaluated using GWAS (Sakiroglu and Brummer, 2017). Results revealed 65 SNPs with significant association with more than one of these traits and five of the candidate were found to be at a close vicinity to the genes involved in growth traits. Yu (2017) genotyped 200 *M. sativa* accessions with a set of 10,327 SNP markers and subsequently performed GWAS analyses for yield under drought stress through. A total of 28 SNP markers with a significant association with yield under drought were found. Biazzi et al. (2017) conducted association mapping for nutritive quality on a panel of 154 plants from a cross between three cultivars and 83 SNP were found to be significantly associated with the nutritive-quality. Liu et al. (2019) applied the GWAS approach to identify loci associated with salt tolerance of 304 accessions using five agronomic and physiological traits and a total of 53 significant SNPs were identified. Wang et al.

(2020) applied the GWAS approach to a collection of 322 *M. sativa* genotypes to investigate marker-trait associations for nine agronomic traits characterized across three consecutive years. They identified a total of 115,654 high-quality single nucleotide polymorphisms (SNPs). Among those, 42 SNP were significantly associated with target traits. Medina et al. (2020) conducted an association mapping for vigor and yield under salt stress in a panel of 304 *M. sativa* accessions. GWAS identified 27 SNP markers associated with vigor and yield under salt stress and six were significantly associated with yield alone. Lin et al. (2021) genotyped 198 accessions of *M. sativa* for drought tolerance for 26 quality traits and a total of 131 markers associated with multiple traits under drought. He et al. (2022) conducted GWAS for salt tolerance related traits using a set of 220 alfalfa varieties. The plants were genotyped with 875,023 SNPs and phenotyped for salt tolerance and germination ability. A total of eight marker traits association were detected. Despite the great success of the GWAS method, it currently has numerous limitations. The major of these limitations is elevated false positives arising from population structure and low-frequency alleles, and detection of non-causative SNPs (Hayes and Goddard, 2010; Liu et al., 2019). SNPs other than true causative polymorphisms can demonstrate more significant associations in GWAS than the causative ones, making it difficult to identify target SNPs. Being an autotetraploid, alfalfa has another challenge in GWAS studies, namely accurate estimation of allele states during heterozygous calls. Accurately identifying genotypes requires high coverage sequencing, increasing the cost of genotyping (Medina et al., 2020). Furthermore, biallelic SNPs are commonly used in polyploids because they are the most common and can be easily converted to numerical form to create genotype matrices. However, approximately 20% of the high-value SNP markers are not biallelic and are discarded during construction of the vcf file. Thus, it is difficult and expensive to achieve the coverage with genome sequencing or GBS approaches to account for tetraploid allele abundance and to distinguish between the three heterozygote classes to perform GWAS (Kumar et al., 2022).

4. CONCLUSION

This review provides an overview of the GWAS approach with its application to identify and characterize the genetic control of agronomic traits in alfalfa and other *M. sativa* populations. A better understanding of genetic control of desired traits in alfalfa will aid breeding efforts. GWAS has successfully identified the genetic factors (significant SNPs) that control phenotypic variation in many traits. Identification of these genetic factors facilitated genetic marker assisted alfalfa breeding. Nonetheless, the identified associations could further be used in newly emerging techniques such as genome editing as well as genomic selection to further accelerate improvement of agronomic performance and nutritional quality of alfalfa and thus to contribute the global food security.

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