

Exploring impact of integrated breeding strategies in enhancing yield, nutritional quality, and stress tolerance in alfalfa

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ABSTRACT

Yield, nutrition quality and stress tolerance are important traits for alfalfa improvement perspective. These qualitative and quantitative attributes are changed during its life cycle, no updated studies available on whether and how these traits are influenced by several environmental factors. Therefore, we updated the role of several breeding strategies for developing alfalfa yield, nutritional quality and biotic-abiotic stress tolerance in alfalfa. This study explored integrated breeding approaches would be suitable for the desire traits improvement in alfalfa. Subsequently, the integration of multiomics including genomics, transcriptomics, proteomics, metabolomics, and ionomics may facilitate the agronomic traits improvement and plant fitness in alfalfa. Furthermore, this study proposes integration of omics-system with top-down (phenotype to genotype) and bottom-up (genotype to phenotype) model that can be helpful to characterize or develop desire qualitative and qualitative traits in alfalfa. This updated study might be useful to alfalfa breeders and farmers for improving alfalfa through breeding programs.

INTRODUCTION

Alfalfa (*Medicago sativa* L.), known as “Queen of the Forages” is an important forage legume. Alfalfa is widely cultivated as a forage crops with good source of protein, and alternative nitrogen source [1]. These positive attributes lead to consider it as ideal crop for livestock and dairy production. Alfalfa is an autotetraploid ($2n = 4x = 32$), allogamous, perennial legume crop cultivated worldwide [2]. Most of the varieties of cultivated alfalfa originated from subsp. *sativa* or subsp. *× varia*, or by its other sub-species hybridization [3]. Forage yield, nutritional quality, and the ability of stress tolerance and/or resistance are key agronomic traits in alfalfa plants. Several qualitative attributes traits such as crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) are important features for ensuring fodder quality of alfalfa [3]. Unfortunately, these traits are either genetically control or influenced by multiple environmental stress stimuli [4]. Several biotic and abiotic stresses significantly inhibit plant growth, decline forage productivity (Table 1), which lead to decline market demand and utilization of alfalfa fodder in livestock industry [5-7]. Therefore, cost effective breeding strategies are highly demandable.

Breeding approaches are exciting tool for improving of yield, quality and stress tolerance in alfalfa [8]. Several traditions to breeding approves are commonly used for improving alfalfa form the ancient time to current era [3].The traditional breeding approaches



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provide excellent output for alfalfa improvement, but these approaches are very time consuming, it may not produce desired results in alfalfa [9]. Therefore, a combined breeding programs of traditional, molecular and modern breeding approaches would be exciting, low cost effective and farmers friendly for improving alfalfa yield, nutrition quality, stress tolerance in alfalfa plants. The modern breeding approach based on molecular marker, multiomics information would be suitable for the improvement of traits associated with forage yield and its quality [10, 11]. The farmers traditionally cultivate local germplasms, which are tolerant to multiple stresses and able to adapt arid and semi-arid environments. Unfortunately, some landrace populations are replaced by high yielding cultivars [12]. The uncontrolled introduction of the alfalfa cultivars occurs erosion of genetic diversity (GD), low sustainability to changing environments, which lead to loss of performance (LOP) and productivity of alfalfa [13].

In these above background scenarios, many modern alfalfa breeders are applying multiomics and gene engineering approaches for developing key agronomic traits in alfalfa without losing their performances [4]. Application of modern breeding approaches have made excellent progress in improving alfalfa desired traits from lab to field levels. In this review, we explored the limitations of traditional breeding for improving agricultural and qualitative attributes in alfalfa, and provided updated understanding on how the modern breeding strategies solve the limitations of traditional breeding in case of alfalfa plants. Further, we emphasized the impact of integrated breeding strategies in enhancing yield, nutritional quality, and stress tolerance in alfalfa.

ENVIRONMENTAL FACTORS INFLUENCING YIELD, QUALITY, AND FITNESS IN ALFALFA

Factors influencing alfalfa yield

Alfalfa yield productivity negatively regulated by several factors including seed yield breeding cultivars, cropping system, growth, crop durability, dry matter production efficiency [14]. Alfalfa yield can also be influenced by fertilization usage, genetic barrier, water use efficiency (WUE)[15]. The production of alfalfa regulated by cropping system including soil depth, drainage system, fertilization, and deficit irrigation [16]. Though alfalfa supports in supplying water in dry season through deep root system. Water is a critical factor where rooting depth (8-30 feet) can vary the amount of water availability as well as dry matter production. Deep soil provides better alfalfa yield compared to shallow soils. However, deficit irrigation declines biological nitrogen fixation that leads to decline alfalfa yield [17]. Application of nitrate fertilization and intercropping system significantly influences the nodule formation, dry matter yield and resource use efficiency in alfalfa [18]. The above studies imply that several factors associated with alfalfa life cycle, are involved in declining yield in alfalfa (Figure 1).

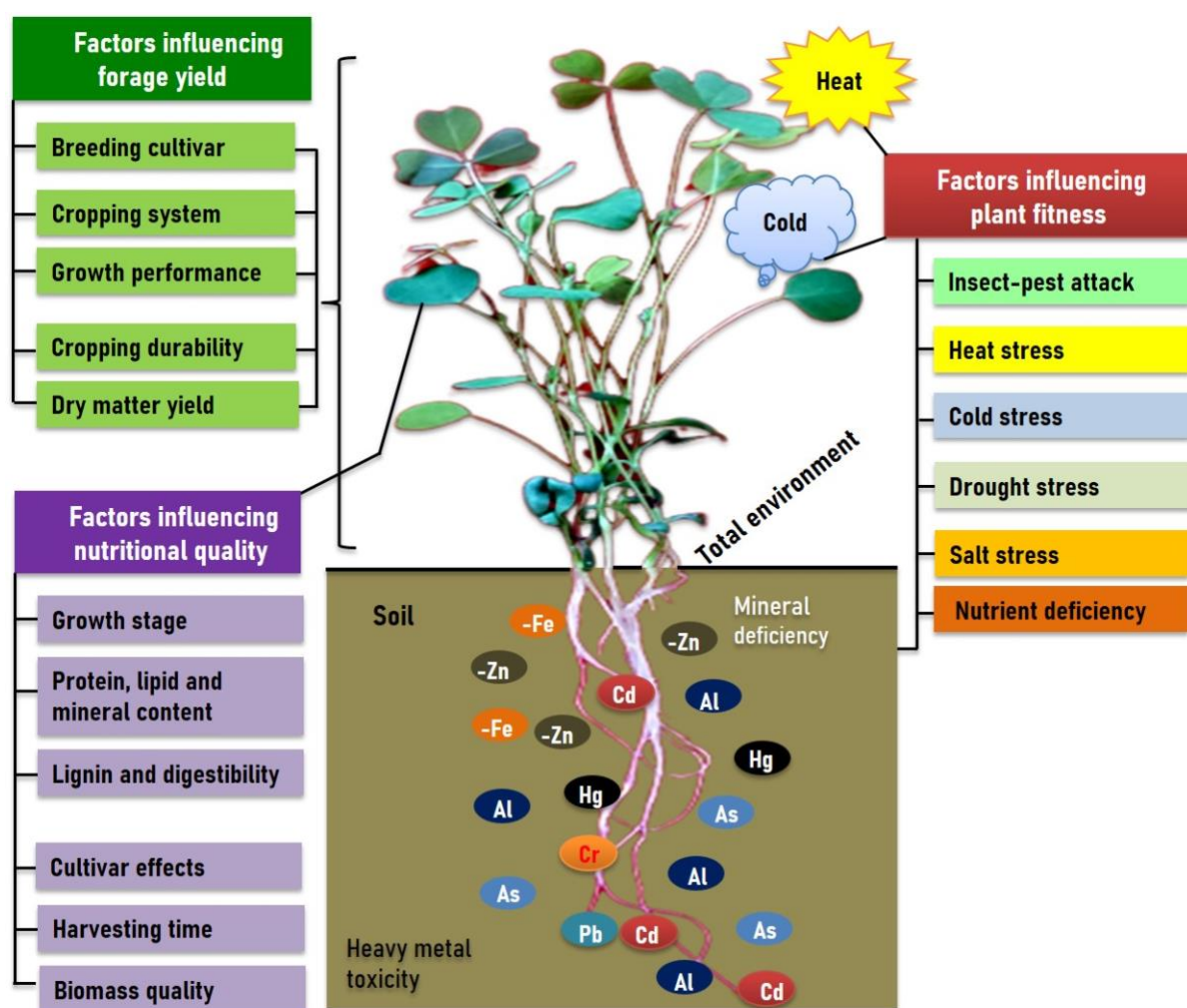


Figure 1. Environmental factors influencing yield, nutritional quality, and plant fitness in alfalfa. Abbreviation, -Zn, zinc deficiency; -Fe, iron deficiency; Al, aluminium, Cr, chromium; Cd, cadmium; Hg, mercury, As, arsenic, and Pb, lead.

Factors influencing alfalfa nutritional quality

Alfalfa is an excellent resources of livestock animals that considered as sustainable source of food proteins [19]. Its nutritional quality alters during plant growth stage and life cycle. The chlorophyll content, amino acids, and flavonoids levels considerably declines at maturity stage compared to other stages in alfalfa [20]. Lignin is a phenolic polymer linked with cellulose, hemicellulose and pectin in the cell wall, high level of lignin influences feed digestibility in livestock animals [21]. As forage crop, alfalfa nutritional status depends on the concentration of carbohydrates, proteins, lipids and present of other phenolic compounds. The arrangement of these organic nutrients decides the digestibility of each crop, along with mineral and mineral values (D-value) and sum of energy that can be obtained by an animal [21]. The accessibility of degrading enzymes to cellulose is restricted by high lignification levels in secondary cell walls; thus, lignin downregulation by genetic modification was used to improve the quality of the alfalfa [22]. Modification of lignin content in alfalfa has been followed in previous studies using both conventional plant breeding methods and transformation methods through lignin-lowering enzymes [22, 23]. The plant harvesting time influences the nutritional quality and silage of alfalfa [24]. Therefore, it is clear that several factors associated with alfalfa

growth, developments are directly or partially involved in regulating nutritional attributes in alfalfa (Figure 1).

Impact of biotic and abiotic factors in alfalfa

Several biotic and abiotic factors including insect pests attack, heat, cold, drought, soil salinity, temperatures, nutrient deficiency are involved in declining growth, productivity and nutrition quality in alfalfa. In the following sections, we stated how abiotic and biotic stress regulates growth, productivity and plant fitness in alfalfa. Insect pests attract under changing climate are critical factors inhibits normal growth, development and plant productivity in the world [25]. The chlorophyll (a, b) content, soluble proteins and dry matter yield significantly declined due to increase of number of thrips attack in alfalfa [26]. This study indicates that interactions among the crops, climate and insects is crucial for damaging crop and yield loss. In this scenario, insect pests can cause more crop damage and productivity loss due to changing climate.

In contrast, multiple abiotic stresses are drastically declined alfalfa growth, yield and nutritional quality in alfalfa (Figure 1). The differences of growing condition significantly influence alfalfa productivity. In a recent study on total 197 alfalfa cultivars suggest that temperature higher than 25°C (>25°C) limits alfalfa growth, and biomass yield [27], while cold stress (<10°C) causes growth retardation, necrosis, decline photosynthetic rate and significant yield in alfalfa [1]. Alfalfa exhibits water scarcity slowly due to deep root system compared to other legumes, but the growth, productivity and protein content considerably declined in response to drought stress in alfalfa [28]. However, stress induced oxidative stress causes cellular injury and even plant death. The high level of salt stress severely causes leaf chlorosis, low photosynthesis performance, decline forage biomass yield and its quality. Previous study reveals that excess sodium toxicity declined plant growth performance, and altered proteomic changes in alfalfa [11].

Mineral deficiency is another key limitation of alfalfa that lead to decline plant growth, fitness, forage biomass yield and its quality (Figure 1). Recently, growth and physiological impairments, and reduction of Zn, S and Ca concentration found in Fe-starved alfalfa [29]. Furthermore, multiple heavy metals (HMs) toxicity severely inhibit plant growth, development and productivity. For instance, the alfalfa growth, biomass yield, mineral nutrition found to be impaired in response to toxic pollutants [6]. Mercury toxicity considerably causes nutrient (Fe, S) reduction in alfalfa [5], while cadmium toxicity causes plant retardation, chlorosis, low photosynthetic performance and biomass yield in alfalfa [30]. Further, Hg toxicity induces oxidative stress in alfalfa that lead to inhibit plants normal growth, photosynthesis and ultimate biomass yield in alfalfa [31]. Therefore, it is clear that mineral deficiency stress along with HMs toxicity directly involved in declining growth, yield and nutritional quality in alfalfa, such type of scenario is presented in case of alfalfa (Figure 1).

REVISITING THE ROLES OF TRADITIONAL BREEDING AND MODERN BREEDING APPROACHES IN IMPROVING ALFALFA TRAITS

Traditional breeding approach

Alfalfa is a autotetraploid ($2n = 4x = 32$) perennial species. Improvement of alfalfa production using traditional breeding is a long term approach, along with several limitations including tetrasomic inheritance, inbreeding depression, and genotype-

environment interaction are always challenging. Despite of these, traditional breeding approach has significant importance for improving desirable traits in alfalfa [32].

Table 1. Impacts of biotic-abiotic stress on alfalfa forage yield and its nutritional quality

Biotic-abiotic stress	Area, Country	Affected traits	Impacts of biotic-abiotic stress	References
<i>Hypera postica</i> (alfalfa weevil)	Oklahoma, Japan	Plant growth, maturity rate, stand density	Decreased growth, maturity rate and stand density (stems/m ²)	[33]
<i>Acyrtosiphon pisum</i> (pea aphid)	Minnesota, USA	DMY, plant height, DDM, maturity rating	DMY, plant height, DDM, maturity rating and economic value of alfalfa are greatly influenced by pea aphid	[34]
<i>Therioaphis maculata</i> (spotted alfalfa aphid)	California, USA	Hay quality, protein and carotene content	Reduced protein and carotene content in damaged hay	[35]
Weevil and aphids	Oklahoma, Japan	FY, stand persistence and TNC	Reduced FY, stand persistence and TNC	[36]
Water deficit	Las Cruces, New Mexico	DMY, WUE, maturity, and leaf/stem ratio	Enhanced early maturity; reduced leaf/stem ratio	[37]
Drought	Becker, Minnesota, USA	FY, ADF, NDF, and ADL	Reduced yield potential by drought stress	[38]
High temperature, CO ₂	Pamplona, Spain	Forage digestibility, quality and yield	Reduced digestibility and CP; enhanced fiber content	[39]
Variable irrigation	Sudan	Plant growth, yield, and WUE	Inhibited plant height, BY, WUE	[40]

ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin; DMY, dry matter yield; FQ, forage quality; TNC, non-structural carbohydrate; FY, forage yield; WUE, water use efficiency; BY, biomass yield; CP, crude protein; DDM, digestible dry matter.

Forage quality has been improved using alfalfa breeding. Milic et al. [41], explored genetically control of important forage quality related traits in leaves and stems of alfalfa genotypes, They found the highest values of narrow sense heritability for NDF and CP in leaves, while ADF was most heritable traits in stems, suggested that nutritive value can be improved using alfalfa breeding program [41]. Study suggests that recurrent phenotypic selection would be effective for improving yield, and tolerance to biotic and abiotic stresses in alfalfa [42]. However, molecular breeding along with other modern breeding approaches have been implemented lately. However, many disease resistance, and pest management, and high yielding cultivars have been developed using these breeding strategies.

Molecular breeding come modern breeding approaches

The significance of QTLs Mapping or gene discovery, marker assisted selection (MAS) and genetic engineering or genetic transformation are emphasised in this section (Figure 2). Alfalfa traits improvement using traditional breeding is time consuming and costly.

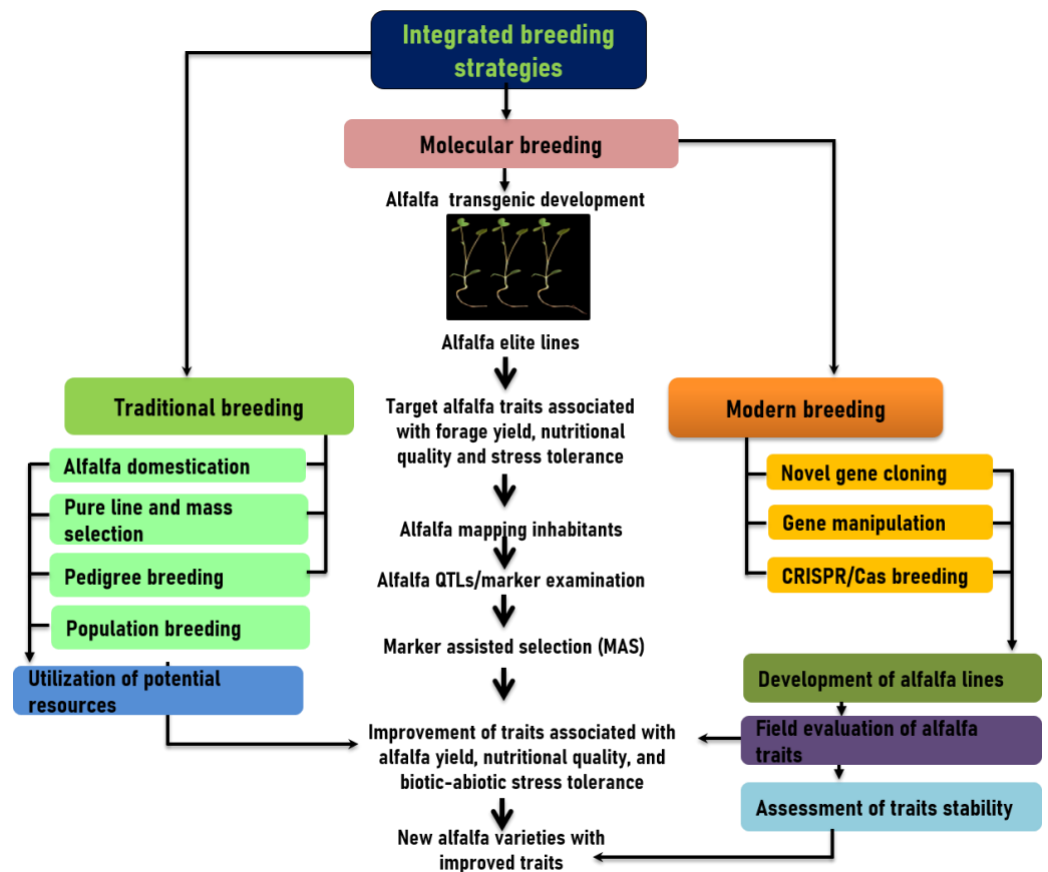


Figure 2. Illustration of integrated breeding strategies for developing traits in alfalfa associated with forage yield, nutritional quality and biotic-abiotic stress tolerance in alfalfa.

Therefore incorporated MAS in to alfalfa breeding programs provide an alternative for alfalfa traits improvement. Though MAS has been minorly applied for commercial improvement of alfalfa compared to other plant species [43]. Identification and development of genetic loci associated with stress tolerance is the initial step of stress tolerant alfalfa variety using MAS. The Quantitative trait loci (QTL) mapping and genome-wide association studies (GWAS) are key approaches for identifying QTLs, which regulate the several traits including yield, forage quality and stress tolerance in plants including alfalfa [43]. The involvement of QTLs associated with forage yield, nutritional quality and stress tolerance in alfalfa (Table 2). Single-nucleotide polymorphism (SNPs) in a region of the genome with high linkage disequilibrium. Total 198 alfalfa accessions were used to SNPs-associated drought stress tolerance by association mapping (AM). Several functional key genes such as *MsHSP23*, *MsZIP*, *MsZEP*, *MsMYB2L*, and *MsWRKY11* associated with drought stress tolerance were identified in alfalfa [8, 43].

In developing modern plant breeding era, gene engineering tool extensively applied in improving insect pests resistance, biotic-abiotic stress resistance in alfalfa (Table 3). These breeding strategies lead to production of novel compounds, improving plant yield, enhancing of industrial and/or pharmaceutical proteins in alfalfa [44, 45]. In modern breeding age, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 approach is being rapidly used for improving alfalfa yield, nutritional quality and stress tolerance in alfalfa plants [46, 47]. The RNAi-mediated down-regulation of alfalfa *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 8* (*MsSPL8*) was involved

in increasing plant branching, enhanced biomass yield, regrowth and drought tolerance in alfalfa [47]. However, despite of the above nutritional and agronomic benefits alfalfa possesses some limitation in usage as pasture and hay forage. A risk factor is initiated specially for tumentents due to high degradation of forage protein. More importantly, high lignin content induces the degradation of carbohydrate in rumen as alfalfa shows an unbalanced and asynchronous degradation of nitrogen to carbohydrate (N/CHO) ration in rumen. In this context, gene engineering tool used for improving nutritive value, yield and performance of stress tolerance in alfalfa [48].

MULTI-OMICS APPROACHES FOR IMPROVING YIELD, QUALITY AND STRESS TOLERANCE IN ALFALFA

High quality of alfalfa is an excellent resource for livestock animals and ultimate global food security. Alfalfa plant fitness, nutritional quality, and productivity dramatically change during its life cycle. The multiomics approach drawn great attention for developing qualitative and quantitative traits in alfalfa (Figure 3). In this section, we updated recent advances and future prospects of multiomic approach in improving alfalfa yield, nutritional quality and biotic and abiotic stress tolerance. A progress of genomics and genomic selection in alfalfa has been stated [10]. Recently, Liu et al. [20] reveal new insights into nutritainal quality alteration in alfalfa leaves during flowering time using multi-omics approaches. They emphasized correlation and network analyses based on leaf metabolome, transcriptome and proteome data, which revealed chlorophyll, flavanoid and amino acid content in alfalfa [20]. Two contrasting alfalfa genotypes have been considered to characterization of salt stress responsive protein using a proteome approach [11]. Alfalfa leagume tolerates drought stress due to deep root system. A series of drough stress responsive proteins has been identified using proteomic approach, which were involved in physiological alteration and drough stress stress tolerance in alfalfa [28]. A combined transcriptome and proteome appaoch has been used for discovering of key factors involved in water logging stress in alfalfa [49], where total 16,899 differentially expressed genes (DEGs) were identified. The abscisic acid and gibberellin-related genes regulation patterns at alfalfa developmental stages analysed by transcriptome approach [50]. These candidate genes were involved in hormone regulation pathway and seed development that opens a new avenue to improve seed yield in alfalfa. Recently, 220 alfalfa cultivars sequences and conducted a genome-wide association (GWA) study using 875023 SNPs [51], while 1,924, 2,405, and 3,779 differentially expressed genes (DEGs) identified at three different growth stages in alfalfa. The GWA combined with transcriptome analyses reveal that genetic loci and key genes lead the flowering time in alfalfa. The series of candidate genes have great significance to breeders for alfalfa traits improvement using breeding programme. However, proper breeding strategies are highly required for desirable traits improvement in alfalfa. Medina et al. [52], yield and vigor traits in response to salt stress in alfalfa, where genotype-by-sequencing (GBS) based marker with allele dose and phenotypic data analysed by GWA. The GWA identified 27 SNPs associated with salt stress tolerance in autotetraploid alfalfa ($2n = 4x = 32$). The GWAS was applied for identifying mapping loci associated with alfalfa growth and forage yield under salt stress [53].

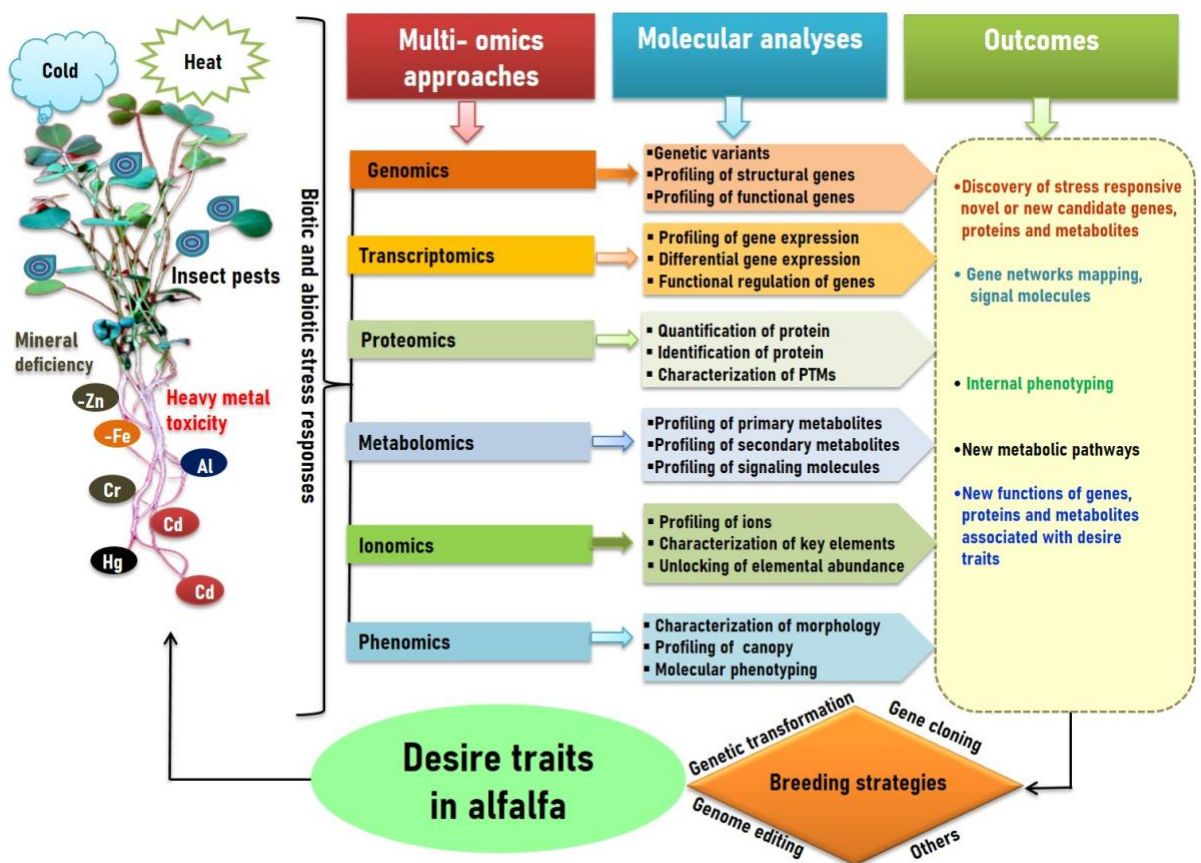


Figure 3. Multi-omics approaches for developing desire traits in alfalfa. Abbreviation, -Zn, zinc deficiency; -Fe, iron deficiency; Al, aluminium; Cr, chromium; Cd, cadmium; and Hg, mercury.

Plant phenotyping plays a key role in alfalfa breeding, along with contribute to explore the link between genotypes and phenotypes. Phenomics-assisted selection approach applied for herbage accumulation in alfalfa [54], where remote sensing technology has been frequently using in alfalfa for herbage accumulation needs for phenotyping efforts such as screening of large breeding population [55]. The application of ionome plays in screening, characterization and distribution of elements in a system. Advances in ionome in alfalfa studies is still elusive. In a study earlier, roots and shoots of seven alfalfa populations screened to examine nickel (Ni) binding ability from aqueous solution, where reported that 80% of Ni bound to the plant tissue and the Ni abundance was 4.1 mg g⁻¹ plant biomass. In contrast, alfalfa was able to remove Ni by 90% from tissues [56]. Isonomic studies have not been well studied in alfalfa. Therefore, this approach can be applied for ions identification, characterization in alfalfa. The above discussions imply the multiomics-tools could be effective for improving yield, nutritional quality and stress tolerance in alfalfa.

The multiomics tool can also be used for new elite genotype development of alfalfa (Figure 4). In this contrast, two contrasting genotype can be considered for addressing population sampling and subsequent molecular analysis including gene and genome sequencing, gene expression profiling, omics data integration, QTLs identification, functional analyses and ultimately screen and/development of new elite plant genotypes with desire traits (Figure 4).

Table 2. QTLs involved in yield, nutritional quality, and biotic-abiotic stress tolerance in alfalfa

Traits of QTLs studied	Number of QTLs	Mapping tool: marker	Mapping population	Ploidy	Map length(cM)	References
Forage yield, drought stress	10-15	LM: SSR, SNP	BC ₁	4x	1049, 1293	[57]
Forage re-growth, yield, and plant height	86	LM	F ₁	4x	--	[58]
Biomass yield	41	LM: RFLP,SSR	F ₁	4x	546	[58]
ADF, NDF, ADL	17	AM:SSR	F ₂	4x	--	[59]
SLR, ADF, NDFD, ADL	83	AM:SNP	F ₂	4x	--	[42]
Lignin modification gene, ADF, NDF, ADL and TNC	7	AM:SSR,SNP	F ₁ ,F ₂	2x	--	[60]
<i>Verticillium</i> wilt resistance	56	AM: SNP from GBS	F ₁	4x	--	[61]
<i>Stagonospora</i> root and crown rot resistance	17	LM: SSR, AFLP,	BC	4x	794	[62]
Anthracnose resistance	33	LM: SSR, AFLP,	BC	4x	--	[63]
Associated with winter hardness, fail growth, and freezing injury	1-6	SMA: RFLP	BC	4x	443	[64]
Aluminum tolerance	7	LM: SSR, RFLP,	BC	2x	764	[65]
Lodging resistance	10	LM: SNP,AFLP,SRAP	BC ₁	4x	1497	[66]

QTL, quantitative trait loci; ADF, acid detergent fiber; NDF, neutral detergent fiber; ADL, acid detergent lignin; SLR, stem/leaf ratio; TNC, total non-structural carbohydrate ;NDFD, neutral detergent fiber digestibility; LM, linkage mapping; AM, Association mapping; SMA, single marker analysis; SNP, single nucleotide polymorphism; AFLP, amplified fragment length polymorphism; RFLP, restriction fragment length polymorphism; GBS, genotyping by sequencing; GWAS, genome wide association mapping; SSR, simple sequence repeat; RAPD, random amplified length polymorphism; SRAP, sequence-related amplified polymorphism; BC, back cross; F₁, first filial generation; 4x, tetraploid ; 2x, diploid

Table 3. Improvement of yield, quality, and biotic-abiotic stress tolerance in alfalfa through molecular breeding

Gene name	Source	Promoter	Performance of transgenic alfalfa	References
AaEβF (E-beta-farnesene)	<i>Artemisia annua</i>	35S-CaMV	Controlled aphid by changing behavior	[67]
s-cryIC (synthetic cryIC gene)	<i>Bacillus thuringiensis subsp. galleriae</i>	35S-CaMV	Conferred Spodoptera pest insect resistance	[68]
ZxNHX and ZxVP1-1 (Cation/H+ antiporters and vacuolar membrane proton pump 1)	<i>Zygophyllum xanthoxylum</i>	35S-CaMV	Produced better yield and good quality forage; enhanced tolerance to salinity, drought and phosphate deficiency	[69]
TPS1-TPS2(rehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase)	<i>Saccharomyces cerevisia</i>	rd29A	Increased biomass yield and accumulation of trehalose; enhanced tolerance to drought, freezing, salt, and heat stress	[70]
Mn-SOD(superoxide dismutase)	<i>Nicotiana plumbaginifolia</i>	35S-CaMV	Improved herbage yield and winter survival	[71]
AVP1(Vacuolar H+-pyrophosphatase)	<i>Arabidopsis thaliana</i>	35S-CaMV	Showed better photosynthesis efficiency; enhanced resistance to salt and drought	[72]
C4H (cinnamate 4-hydroxylase), C3H(shikimate 3-hydroxylase), F5H (ferulate 5-hydroxylase)	<i>Medicago truncatula, M. sativa</i>	PAL2	Significantly reduced lignin without significant impact on composition; improved forage digestibility	[73]
CS (citrate synthase gene)	<i>Pseudomonas aeruginosa</i>	Act2, RB7	Enhanced tolerance to aluminum (Al) stress	[74]
bar (bialaphos resistance gene)	<i>Streptomyces hygroscopicus.</i>	35S-CaMV	Exhibited resistance to the herbicide glufosinate-ammonium [ammonium -DL-homoalanin-4-yl (methyl) phosphinate]	[75]
MsHsp23 (heat shock protein)	<i>Medicago sativa</i>	35S-CaMV	Enhanced tolerance to oxidative and heat stress	[8]
lbor (orange gene)	<i>Ipomoea batatas</i>	SWPA2	Increased carotenoid accumulation; enhanced tolerance to drought and salt stress;	[76]

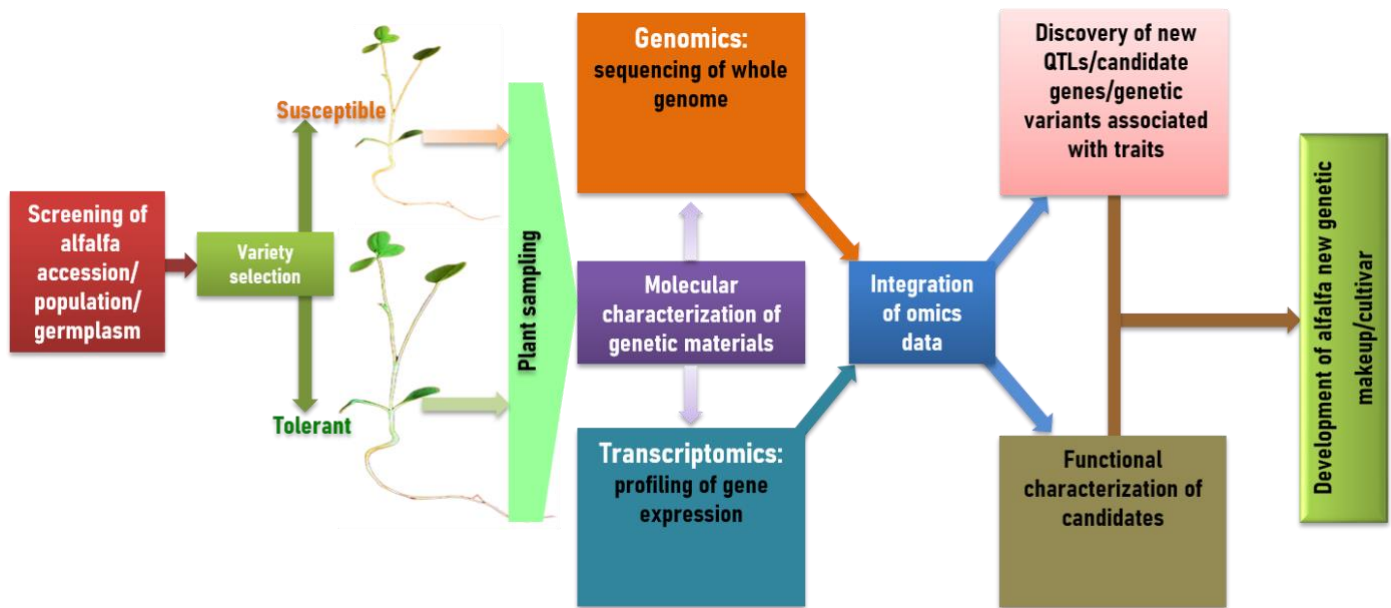


Figure 4. Illustration of working model for developing new alfalfa cultivar from unutilized alfalfa germplasm. Abbreviation, QTL, quantitative trade loci.

CONCLUSIONS AND FUTURE DIRECTIONS

Improvement of qualitative and quantitative traits in alfalfa plants is critical for its overall improvement. This study provides undated insights into the impact of integrated breeding strategies in enhancing yield, nutritional quality, and stress tolerance in alfalfa. We have described significance of molecular breeding including gene engineering or manipulation approaches for improving new traits and plant fitness in alfalfa plants. Additionally, the potential role of integrated traditional breeding and modern breeding approaches in improving yield, nutritional value and biotic-abiotic stress tolerance in alfalfa. Though such breeding strategies has already been implemented in several alfalfa species but there is still scope to improve alfalfa traits using multiomics molecular tools. Furthermore, the integration of panomics tool combined with multiomics approaches (genomics, transcriptomics, proteomics, metabolomics, phenomics, and ionomics) would facilitate to identify novel/new candidate genes, proteins, phenotypes and metabolites to develop desire traits and plant fitness in alfalfa. The above advances will be useful to alfalfa breeders and farmers for developing new alfalfa cultivars with improved yield, nutritional quality and stress tolerance.

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AUTHORS CONTRIBUTION

AKA, and AM conceived the research plan. AKA wrote initial draft. SKB, AK revised and edited the manuscript. All the authors approved the final version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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