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RESEARCH ARTICLE
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# Creating Original Forms of Alfalfa for Adaptability based on the Hybridization of *m. tianschanica vass*. x *M. sativa L*. in a System of Backcrosses

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## ABSTRACT

Amid climate change and a deficit of irrigation resources, alfalfa selection faces the challenge of increasing adaptability to abiotic stresses, particularly drought and salinity. This study presents the results of a breeding program aimed at developing source forms of alfalfa through backcross hybridization of the wild species Medicago tianschanica Vass. with cultivated Medicago sativa L. Ecotypes differing in corolla coloration and morphological traits were collected from the West Tian Shan and involved in a multi-stage backcrossing scheme with progressive saturation of the progeny with M. sativa L. genes. Field evaluation was conducted under medium chloride–sulfate salinity conditions (1.0–1.5%) in the Kyzylorda Region of Kazakhstan. Out of 138 tested backcross-derived lines, 27 lines (19.6%) demonstrated high salt tolerance, maintaining plant spareness below 20% while preserving stable green mass and seed productivity. Backcrossing resulted in a reduction of seed hardness from 18.4–24.3% in the original wild forms to 6.8–10.1% in the BC3 generation, alongside an increase in erect bush forms and favorable pod morphology. The novelty of this study lies in the systematic use of *M. tianschanica* as a donor of adaptive traits under saline field conditions combined with phenotypic marker-based selection in successive backcross generations. The selected lines represent valuable source material for synthetic breeding programs aimed at developing alfalfa varieties with enhanced adaptability to salinity and arid environments.

**Keywords:** Alfalfa species; Adaptation; Backcrossing; Backcross progeny; Selections.

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## INTRODUCTION

Alfalfa is a leading fodder crop in Kazakhstan and other countries (Bastaubayeva et al., 2023) owing to its fodder and agronomic importance (Dervishi et al., 2023; Selim et al., 2024). This crop is also multifunctional, as the expansion of its area provides ecological effects, contributing to the reduction of erosion processes, the conservation of carbon dioxide in the soil and the accumulation of biological nitrogen in the soil (Nasiyev et al., 2023; Naz et al., 2024). In recent years, farmers in the traditional zone of alfalfa cultivation have been experiencing a deficit of irrigation water. Alfalfa suffers greatly from this deficit, and its potential productivity is not realized: instead of 3–6 harvests, it is cut only 1–2 times (Kuandykova et al., 2024). In these circumstances, it is important to promote selection for drought and salt tolerance (Humphries et al., 2021; Serikbay et al., 2024). Wild alfalfa species growing in the more arid conditions of the natural flora of Kazakhstan continue to provide a genetic source for increasing drought tolerance.

According to the classification proposed by Lubenets (1953), alfalfa subgenus Falcago (Reichb Gross) includes 21 species, of which the diploid species (2n=16) *M. quasifalcata Sinsk., M. difalcata Sinsk., M. coerulea Less.,* and *M. trautvetteri Sumn.* and the tetraploid (2n=32) *M. sativa L. sybsp. Transoxana*, *M. varia Mart.*, *M. falcata L.*, and *M. tianschanica Vass* occur in Kazakhstan. There is an incredible variety of forms in populations among recognized species in the places where they grow naturally, in the primary foci of cultural origin and evolution. This diversity has been divided by botanists and breeders into ecotypes of species (in terms of growing conditions) and biotypes within populations without

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regard to conditions (Sánchez-Reyes & Fernández-López, 2024). Given the escalating impact of climate change on agriculture and food security, it is safe to hypothesize that the adaptability of forage crops to climatic change is critical to safeguarding livestock, protecting land mass, and maintaining food security. Thus, alfalfa adaptation to climate stress can be classified as a global issue. Zhang et al. (2024), to better understand alfalfa's climatic adaptation and genetic susceptibility to future climate change, conducted population genomic studies utilizing genome resequencing data from 702 accessions of 24 Medicago species. They discovered that interspecific genetic exchange contributes to the alfalfa gene pool, particularly by increasing defense and stress response genes. They also advocated for targeted conservation and breeding activities. Although thorough, this study could be hampered by the very complex demographic history of the populations, such as population bottlenecks, expansions, and unequal gene flow, as well as the absence or neglect of microclimates due to the resolution of environmental capture. Guo et al. (2024)'s review explained from numerous studies that the alfalfa's migration from West Asia has varying effects on genetic diversity across regions.

Introgression from the subspecies *falcata* increased genetic diversity in populations across Central Asia, East Asia, and Europe/North America. In contrast, populations in Africa and South America experienced a decline in variety. High levels of introgression in populations can lead to more harmful mutations, highlighting the possible consequences of genetic exchange (Zhang et al., 2024; Ahad, 2025). Wang et al. (2023), in their study across four long-term grazing zones in western Canada, discovered that the GEAs (genotype-environment associations) that were identified by genotyping-by-sequencing (GBS) data of the alfalfa populations and environmental data were able to find 31 SNPs related to 13 candidate genes that were mostly connected with six environmental parameters, among which candidate genes underlying environmental conditions were linked to a wide range of proteins implicated in plant responses to abiotic stresses such as drought, cold, and salinity-alkali. This research is hampered by the inverse relationship of correlation and causality, and more comprehensive studies are still required. Annicchiarico et al. (2022), in their multicontinental assessment using genomic selection ability to forecast alfalfa breeding potential for drought-prone agricultural sites in Algeria, Morocco, Italy, Tunisia, and Argentina, concluded that specific breeding for each target region highlights the important role genomic selection plays in most areas when considering the challenges associated with phenotypic selection.

Kazakhstan stands out from many other countries with its diversity of wild alfalfa, which includes seven species and numerous ecotypes growing in more arid conditions with traits of resistance to drought, salts, diseases, and a tendency to longevity (Meiirman et al., 2017; Meiirman & Yerzhanova, 2015; Abaev et al., 2023a). They have developed traits conducive to the survival and conservation of the species through natural reproduction (self-seeding). Thus, these species and ecotypes present a valuable natural source for enhancing the adaptability of cultivated varieties (Koptileuov et al., 2024). Some wild species, such as *M. tianschanica Vass.* and *M. sativa L.,* have a very diverse biotypic composition, within which intraspecific natural hybridization occurs with the emergence of various introgressive forms, which can be distinguished by corolla coloration and used in selection. *M. tianschanica Vass.,* which has been recognized as a stand-alone species (Ivanov, 1980), introgresses with other species and evolves as a population with a complex genetic structure, often referred to as *M. varia Mart.* In the phylogenesis of the genus *Medicago* from diploid to tetraploid species, *M. tianschanica Vass.* is considered a young hybrid species that became the immediate progenitor of domesticated alfalfa.

The primary center of origin of the tetraploid alfalfa species *Medicago sativa* L. and *Medicago tianschanica* Vass., as proposed by Zhukovsky (1968), is the Eurasian gene center. Wild alfalfa ecotypes from the Central Asian gene center represent a valuable genetic reservoir for the development of drought-tolerant cultivars, owing to their pronounced xerophytic adaptations. These adaptive traits include the presence of trichomes on above-ground organs, stem pigmentation, a strongly spreading and occasionally prostrate or ascending growth habit, lanceolate leaves, reduced leafiness, and a relatively thin yet deeply penetrating root system characterized by well-developed taproots (Ivanov, 1980; Yu et al., 2017; Jasim & Sultan, 2023).

In the Middle Asian gene center in the plain belt, the foothills and lower tier of mountains are widely populated by *M. sativa L. sybsp. Transoxana*. The lower belt of mountains is occupied by *M. falcata L.*, and the intermediate belt of the Tian Shan is occupied by *M. tianschanica Vass.* — A species of hybrid origin with a phenotype of either blue (sativa type) or yellow corolla and sickle-shaped pods (falcate type) (Vasilchenko, 1938). *M. tianschanica Vass.* also shows signs of disease resistance and drought tolerance. The main area of its localization is the West Tian Shan (Kazakhstan). Vasilchenko (1938), the author of the *M. tianschanica Vass* taxon, describes the species as follows: corollas 8–10 mm long, variously colored (blue-violet, purple, brown, blue-greenish, dirty pink, etc.); pods coiled in one or two turns.

However, despite this genetic potential, systematic backcrossing of M. tianschanica under saline field conditions remains insufficiently studied. In the natural flora, *M. tianschanica Vass.* occupying the plain part of the Tian Shan Mountains became the ancestor of the cultivated species *M. sativa L.* through *M. sativa L. sybsp. Transoxana* during species evolution. The local population has used this wild species for centuries for fodder production and introduced it with the selection and propagation of sativa-type plants, i.e., blue-flowered forms, with different shades of the corolla coloration, excluding yellow ones. Centuries of folk breeding resulted in the accumulation of the traits characteristic of *M. sativa L.*

today: regrowth capacity, ability to produce multiple harvests, a high rate of spring and post-cutting regrowth, pod turnover (up to 2.5–4.0 revolutions), and erect or sprawling bush forms (Selim et al., 2024). Thus, yields gradually increased, and the local population later labeled the resulting species as Turkestanian. *M. tianschanica Vass.* also served as the germplasm for many alfalfa varieties in Europe and New World countries. To apply *M. tianschanica Vass.* in the selection program to increase the adaptive capacities of *M. sativa L.* varieties, expeditions were carried out to collect specimens of *M. tianschanica Vass.* and other alfalfa species (Methodological instruction, 1981; Painting, 1996; Abaev et al., 2023b) also included in the selection process through backcrossing. This program aimed to improve drought tolerance in cultivated varieties in the face of climate change and water scarcity by transferring the desired resistance genes (Ilyushin & Martirosyan, 2024; Kazankapova et al., 2024). The transfer of drought tolerance genes into modern alfalfa varieties at the present stage of scientific progress is possible through single hybridization with a donor and the displacement of other negative traits in hybrid generations using systematic backcrossing for at least 3–4 generations (Dey & De, 2023; Yazid et al., 2024).

By introgressing resilience traits from a wild relative into a cultivated species, this study hopes to improve alfalfa adaptability in the face of climate change and water scarcity. The main hypothesis is that progeny with a restored cultivated phenotype while retaining important adaptive genes would be produced by systematically backcrossing *M. tianschanica Vass.*, a donor of drought and salt tolerance, with *M. sativa L*. The program validated the method for producing superior source material for synthetic alfalfa varieties by successfully developing lines that combined high productivity and salt resistance with desirable agronomic characteristics through phenotypic selection for marker traits.

Therefore, the objective of this study was to develop and evaluate backcross-derived alfalfa lines from Medicago tianschanica Vass. × Medicago sativa L. with enhanced adaptability to saline and arid conditions, using phenotypic marker traits and field-based assessment of productivity and salt tolerance.

## MATERIALS & METHODS

### Specimen Collection

Wild alfalfa species — the source material for hybridization — including ecotypes of *M. tianschanica* Vass. were collected from natural landscapes in the transition area from the Tian Shan Mountains to Karatau (South-East Kazakhstan). A map of the study area, including collection sites and the field evaluation location, is presented in Fig. 1.

The expedition collected 16 natural ecotypes of *M. tianschanica* Vass. species, differing by the marker traits of the corolla flower coloration and bean turnover. Plants forming the structure of ecotypes in the conditions of the introduction nursery (in culture) were also classified by corolla coloration and bean features. In the process of backcrossing, i.e., the saturation of hybrid progeny with *M. sativa* L. genes, individuals similar to *M. sativa* L. were selected from among the various divergent hybrid variants, controlling the expression of a set of traits: bush type, corolla color, pod turnover, leaf shape, regrowth after cutting, and seed hardness.

### Study Materials

Cultivated alfalfa varieties belong to two botanical species: *M. sativa L.* and *M. varia Mart.* M. *sativa L.* varieties regrow fast, have high yields, grow intensively with irrigation, provide 3–6 harvests, respond well to fertilizers (Asenov et al., 2023), can produce up to two harvests on dryland, and are cultivated mainly in South-East Kazakhstan. *M. varia Mart* varieties are winter-hardy and cultivated in northern regions under non-irrigated farming conditions, where they produce only one, sometimes two harvests due to the short summer period. *M. tianschanica Vass.* is an evolutionally young hybrid species. It has been comprehensively studied by botanists from Central Asia, such as Vasilchenko (1948), L.V. Dmitrieva (1958, 1967) and Kultiasov (1967), whose research has not lost relevance to the present day. The parental forms chosen for hybridization included 15 species, divided into several groups. The first group consisted of plants with blue-greenish corollas and pods with 1–2 revolutions. The second group included plants with dirty yellow coloration of the corolla and pods with up to one turnover.

**Fig. 1:** Location of the study area.

In addition, we identified the third group of plants distinguished by blue-violet corollas and 2.5 or more pod turnovers. They were not included in the program of hybridization with cultured alfalfa, because it would have been impossible to determine the degree of hybridization based on the color of the corollas in backcross generations. This group of *M. tianschanica Vass.* plants had little difference from *M. sativa L.* Studies with these plants were continued in other variants of selection work. In the process of hybridization, the identified *M. tianschanica Vass.* plants were used as female parent plants and donors of drought and heat tolerance, disease resistance, and salt tolerance. The recipient plants belonged to the variety Semirechenskaya local (*M. sativa L.*).

Artificial crossing without flower castration were performed with at least 100 flowers from each plant, marking the inflorescences (racemes) with a small tag inscribed with the number of opened and pollinated flowers. This approach ensured uniform hybridization intensity across parental combinations.

### Research Stages

Wild alfalfa *M. tianschanica Vass.* participated in hybridization in the original variant. Next, hybrid progeny was saturated with *M. sativa L.* genes through backcrossing up to the 4th generation. Continuous backcrossing was performed to increase hybrid productivity by displacing (eliminating) undesirable traits inherent in wild alfalfa species, such as the recumbent bush type, low leafiness, high seed hardness, and little regrowth after cutting. At the first stage of the study, plants in nurseries intended for hybridization were planted in 60x30 cm crops. Parental forms for hybridization were selected by phenotype, visually based on bush vigor and regrowth in spring and after cutting, relying on the marker trait of blue-floweredness. At the second stage, adult buds were first isolated with a gauze insulator to increase the number of flowers ready to open. Then, at the moment before flower opening, the opened flowers of the male parent were placed next to the vexillum (corolla) of the female parent. Due to the biological mechanism of alfalfa flower opening, pollen transfer occurred without artificial castration, resulting in successful hybrid seed formation. After crossing, the isolators were removed because bees do not visit already opened flowers. At the next stage, to evaluate the success of hybridization, the following parameters were documented: degree of bean setting, seed yield per bean, changes in corolla coloration and bean turnover (number of revolutions), bush type, leaf shape, seed hardness, and productivity in fodder mass and seeds.

Following this, the later generation of backcrosses ($BC_4$) of *M. tianschanica* and *M. sativa L.* were tested under hot climate conditions (Kyzylorda oblast, Kazakhstan) against the background of medium chloride-sulfate salinization (Hoshan, 2024). Soil salinity at the experimental site was of natural origin and ranged from 1.0–1.5%, determined by laboratory analysis of soil samples collected from the 0–30cm layer prior to planting. Field evaluation was conducted using a randomized complete block design with three replications. Each backcross-derived line was represented by plots of uniform size, and standard agronomic practices recommended for the region were applied uniformly across treatments. The plants were analyzed based on agronomic indicators: development phases, productivity of fodder mass and seeds, and resistance to the salt factor.

### Data Processing

Statistical processing of the experimental data used analysis of variance (ANOVA) and the chi-squared test and was performed in RStudio. The ANOVA model included genotype (selection line) as the main factor, and differences were considered statistically significant at $p \leqslant 0.05$.

## RESULTS & DISCUSSION

During our expedition in Kazakhstan (Methodological instruction, 1981), we accumulated a large collection of wild alfalfa (144) of various ecotypes within the seven available species, including *M. tianschanica Vass* 16 samples were collected in the West Tian Shan and Karatau Mountains. In a preliminary study of wild species in cultured conditions (in the introduction nursery), the relative average level of green mass productivity compared to *M. sativa L.* (cultivated alfalfa, Semirechenskaya local variety) amounted to:

a) in 25 specimens of *M. varia Mart.* — 93.2±3.3
b) in 12 specimens of *M. sativa L. sybsp. Transoxana* — 86.0±2.4
c) in 59 specimens of *M. falcata L. — 80.3 ±2.*5
d) in 16 specimens of *M. tianschanica Vass.* — 75.6±3.0
e) in 20 specimens of *M. difalcata Sinsk.* — 49.0±3.1
f) in 10 specimens of *M. trautvetteri Sumn.* — 40.6±2.9
g) in 2 specimens of *M. coerulea Less.* — 38.1±2.2 (Kalibayev et al., 2021).

These data show that tetraploid species are more productive than diploids under cultivation conditions. These information aligns with relevant studies on alfalfa ploidy dating back to as far as 1975, it also aligns with the Pasquali (2024) study on focused on Italian ryegrass (*Lolium multiflorum Lam.*) and alfalfa (*Medicago sativa L.*) in which he concluded that polyploid plants gain many advantages compared to diploid plants, especially in terms of vigor and adaptability. Contrary to expectations, İlhan (2022) maintains that there is a scarcity of regional data on tetraploid alfalfa species thereby making it insufficient to make conclusive remarks or comparative analysis between tetraploid and diploid species of the alfalfa population. In the process of phylogenesis in wild species, evolution took the direction of accumulating adaptive traits and properties, such as drought resistance, winter hardiness, deep position of the root neck (crown) in the soil, seed hardness, recumbent type of bush, prolongation of the flowering period, and pod cracking (some have straight pods). In addition, evolution proceeded from yellow-flowered to blue-flowered and, in conjunction with this, from straight pods to curled pods and from the recumbent bush type to the erect type. This trend has been experimentally confirmed in backcrosses of *M. falcata L.* and *M. sativa* L. (Meiirman et al., 2022, 2024). The evolutionary rend of alfalfa plants aligns with the studies of Medina et al. (2025) and Liu (2025) who observed and documented key adaptability traits in specific ecosystems. Liu (2025) highlighted that some alfalfa grown in saline soils developed salt resilience while Medina et al. (2025) highlighted that following an analysis of alfalfa in the northern US latitude, the introduction of *Medicago sativa sp. falcata* into *Medicago sativa ssp. sativa* has been observed to bring frost resistance. Notably, In the backcross generations, phenotypic marker traits (such as corolla color, and pod shape) served as the basis for the entire selection process. Future research should include molecular markers like SSR and SNP to verify the true introgression of the target genes from the donor and recipient, even though these are helpful visual indicators. Certain lines may have the desired phenotype but lack the underlying genetic resilience, and the opposite may be true.

The use of phenotypic marker traits, including corolla coloration, pod turnover, bush type, and seed hardness, proved effective for monitoring the degree of introgression and selection progress in successive backcross generations. These traits have long been recognized as reliable indicators of genetic background and agronomic suitability in alfalfa breeding and allow rapid, cost-effective screening of large hybrid populations under field conditions (Ivanov, 1980; Humphries et al., 2021). While molecular tools provide additional resolution, phenotypic selection remains a practical and widely applied approach at early stages of pre-breeding, particularly when working with extensive material under stress environments. The species included in the crossing had the same tetraploid set of chromosomes in the karyotype (2n=32). Despite many visible morphological differences, they hybridize freely. The conjugation of chromosomes (pairing) was very successful, as proven by the level of seed bean setting (Table 1). The resulting level of bean (seed) setting is not inferior to that of intervarietal hybrids of *M. sativa L.* under artificial hybridization. In the original variant of *M. tianschanica Vass.* x *M. sativa L.,* bean setting amounted to 60.3–64.7% with 1.5–1.8 seeds per bean. As the genes of the parent *M. sativa L.* became more prevalent in backcrosses, bean setting and the yield of seeds per bean increased. This increment was observed in variants with two different types of donors from the population of *M. tianschanica Vass.*: a) plants with a blue-green corolla and b) plants with a dirty yellow corolla. The first variant demonstrated somewhat higher indicators than the second, which suggests that blue-flowered plants were closer to *M. sativa L.* In the backcrossed generation ($BC_3$), bean setting reached 77.3–79.1% with 2.3–2.5 seeds per bean. In the framework of creating source forms with wild species and using them for synthetic selection, the parameters of hybridization capacity and bean setting play a critical role in the internal integration of populations. The degree of saturation of the *M. tianschanica Vass.* x *M. sativa L.* hybrid combination throughout backcrossing with *M. sativa L.* was analyzed based on marker traits: the color of the corolla, pod turnover, bush type, leaf shape, and seed hardness. In each subsequent round of backcrossing, the selected plants were those with the marker traits characteristic of *M. sativa L.* In the original crosses of *M. tianschanica Vass.* x *M. sativa L.* hybrids, blue flowers and other quantitative traits associated with it (spiraled pods, sprawling and erect bush type, and ovate leaves) were predominant and increased in subsequent backcrossed generations. This process was also influenced by the selection of blue-flowered plants, as they were used as parents for further backcrosses.

In the $BC_1$–$BC_3$ backcross offspring from the original parent plant (donors) with blue-colored corolla (*M. tianschanica Vass.*), the share of blue-flowered plants increased from 43.3 to 75.3%. Accordingly, the proportions of other corolla colorations dropped — from 26.7 to 8.3% for variegated and from 30.0 to 16.4% for violet (Table 2). A similar pattern was observed with the original parent plants with dirty yellow corollas. In this case, the share of $BC_3$ plants with blue flowers reached 56.3%, and the share of individuals with violet corolla ranged from 27.4 to 33.4%. Different shades of blue and violet corolla are frequently observed in variety populations of *M. sativa L.* Plants in the $BC_1$–$BC_3$ generations collectively referred to as "purple" had this kind of the corolla coloration, including intermediate variants with different tints: white, dirty yellow, light yellow, blue-greenish, dirty pink, brown, etc. As saturation with *M. sativa L.* genes increased, the number of these plants grew, and they were excluded from the experiment during backcrossing.

**Table 1:** Bean and seed set in the artificial crossing of M. *tianschanica Vass.* x *M. sativa* L.

| Biotype *M. tianschanica Vass.* | Original cross | | Backcross | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | $BC_1$ | | $BC_2$ | | $BC_3$ | |
| | Bean set % | Seed yield per bean | Bean set % | Seed yield per bean | Bean set % | Seed yield per bean | Bean set % | Seed yield per bean |
| Plants with a blue-green corolla flower size | 64.7±3.5 | 1.8±0.13 | 71.2±5.0 | 2.2±2.0 | 73.5±4.2 | 2.6±3.0 | 77.3±–4.0 | 2.5±0.40 |
| Plants with a dirty yellow corolla flower size | 60.3±3.0 | 1.5±0.20 | 66.4±3.3 | 2.1±2.0 | 69.7±2.4 | 69.7±2.3 | 79.1±5.0 | 2.3±3.5 |

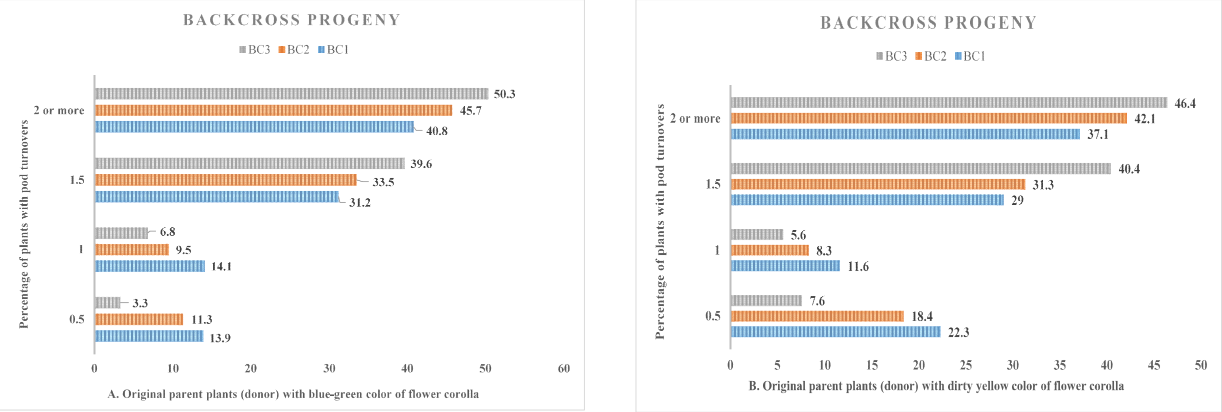
**Table 2:** Manifestation of the "corolla color" marker trait in offspring ($BC_1$–$BC_3$) from backcrossed crosses of *M. tianschanica Vass.* x *M. sativa* L.

| Backcross progeny | Percentage of plants with the color of the corolla | | |
|---|---|---|---|
| | Variegated | Violet | Blue |
| Original parent plants with a blue-green corolla | | | |
| $BC_1$ | 26.7 | 30.0 | 43.3 |
| $BC_2$ | 12.3 | 19.8 | 67.9 |
| $BC_3$ | 8.3 | 16.4 | 75.3 |
| Original parent plants with a dirty yellow corolla | | | |
| $BC_1$ | 30.1 | 27.4 | 42.5 |
| $BC_2$ | 23.6 | 29.7 | 46.3 |
| $BC_3$ | 10.3 | 33.4 | 56.3 |

Note: The variegated coloration of the corolla flowers includes intermediate variants of the corolla coloration with different shades: white, dirty yellow, light yellow, blue-greenish, dirty pink, brown, etc.

The analysis of additional traits associated with corolla color (Fig. 2A, B), including pod turnover (Fig. 2A, B) and bush growth habit (Fig. 3A, B), indicates that increasing introgression of *Medicago sativa* L. genes—characterized by 2–3 pod turnovers and an erect growth form—led to a higher frequency of these agronomically desirable traits within the structure of the segregating backcross populations. Seed hardness, which occurs at approximately 15% in cultivated varieties of *M. sativa* L., is considered during laboratory germination assessments in accordance with the relevant GOST standards, where hard seeds are classified as viable. Elevated seed hardness is typical of wild alfalfa species and contributes to their persistence under extreme environmental conditions, as such seeds can remain dormant in the soil for extended periods and germinate only when conditions become favorable. However, this characteristic is undesirable in agricultural production due to uneven germination. Backcrossing significantly reduced seed hardness: in one case, it declined from 18.4% in the original material to 6.8% in the $BC_3$ generation, and in another from 24.3% to 10.1% ($BC_3$), depending on the initial *M. tianschanica* Vass. genotype. These results demonstrate the effectiveness of the backcrossing strategy in improving seed quality traits (Table 3).

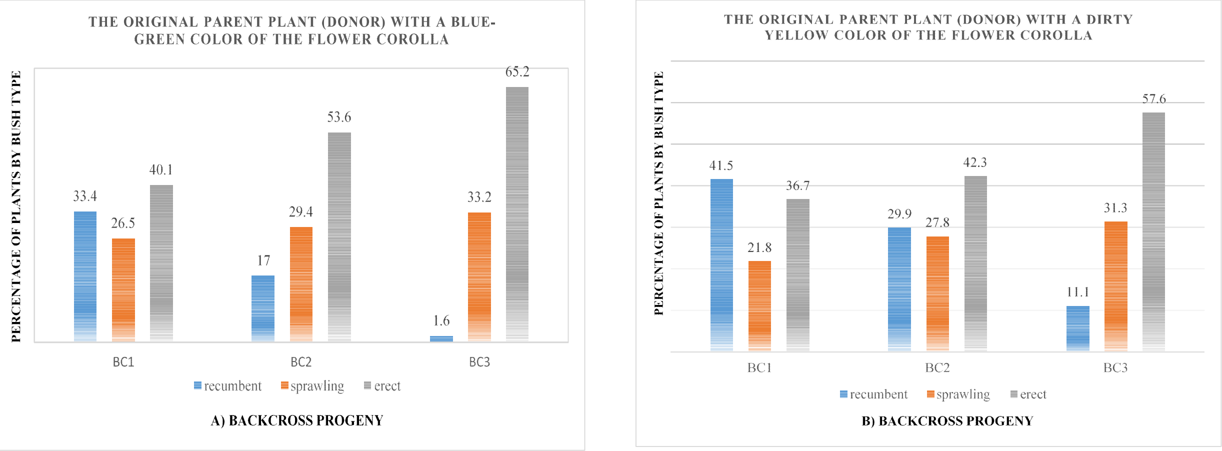
The reduction of seed hardness observed by the BC3 generation is consistent with expected genetic trends during systematic backcrossing to cultivated M. sativa L. High seed hardness is a characteristic adaptive trait of wild alfalfa species, ensuring delayed germination under unfavorable natural conditions; however, it is undesirable in agricultural production. Progressive displacement of this trait through repeated backcrossing and phenotypic selection has been reported in previous interspecific hybridization studies involving Medicago species (Meiirman et al., 2022; Meiirman et al., 2024). The decrease



X-squared = 11.188, df = 6, p-value = 0.08274

X-squared = 12.532, df = 6, p-value = 0.05109

**Fig. 2:** (A, B) — Manifestation of the "bean turnover" marker trait in offspring ($BC_1$–$BC_3$) from backcrossed crosses of *M. tianschanica Vass*. x *M. sativa L.*



X-squared = 35.899, df = 4, p-value = 3.036e-07

X-squared = 23.97, df = 4, p-value = 8.099e-05

**Fig. 3:** (A, B) — Manifestation of the "bush type" marker trait in offspring ($BC_1$–$BC_3$) from backcross crosses of *M. tianschanica Vass*. x *M. sativa L.*

**Table 3:** Manifestation of the "seed hardness in %" marker trait "bean turnover" marker trait in offspring from backcrossed crosses of *M. tianschanica Vass* x *M. sativa L.*

| Biotype *M. tianschanica Vass.* | Original plant | | Backcross generation | | |
|---|---|---|---|---|---|
| | *M. tianschanica Vass* | *M. sativa L.* | $BC_1$ | $BC_2$ | $BC_3$ |
| Plants with a blue-green corolla flower size | 18.4 | 2.7 | 15.3 | 12.6 | 6.8 |
| Plants with a dirty yellow corolla flower size | 24.3 | 2.7 | 18.3 | 15.2 | 10.1 |

in seed hardness to levels comparable with cultivated alfalfa indicates successful recovery of agronomically favorable traits while maintaining stress tolerance. A further limitation of this study is the significant association of blue corolla color with desirable agronomic traits such as erect bus structure and high bean pod turnover. Although this correlation serves as a selection criterion, the fundamental genetic or physiological mechanism behind this examination is yet to be examined, thereby precluding any causal inference. A selection procedure reliant solely on this metric (a singular marker characteristic) may unintentionally exclude potential useful genetic combinations or input unwanted dormant traits that do not necessarily exhibit the blue corolla phenotype. More research is recommended to understand the genetic link between blue corolla color and desirable agronomic traits.

The selected lines from the $BC_3$ generation of the *M. tianschanica Vass* x *M. sativa L.* cross were tested in the Sub-Aral area (Kazakhstan) in a hot climate on medium-salinity chloride-sulfate soil with a salt content of 1.0-=1.5% (Nassima et al., 2024). Out of the 138 tested selection lines, 66 suffered a lot of thinning (over 60%) and 45 perished completely due to the salt factor. Based on salt resistance, 27 lines (spareness up to 20%) were selected from among those that performed best in terms of fodder mass and seed productivity. These lines were similar to *M. sativa L.* in their habitus and marker traits (flower coloration and bush type) (Table 4).

The proportion of salt-tolerant genotypes identified in this study (27 out of 138 lines, 19.6%) can be considered relatively high for field conditions characterized by medium chloride–sulfate salinity and elevated temperatures. In similar studies involving the introgression of wild Medicago germplasm into cultivated alfalfa, the proportion of stress-tolerant progeny retained after early selection stages is often substantially lower, particularly under natural salinity backgrounds rather than controlled environments (Humphries et al., 2021; Meiirman et al., 2024). The observed survival and performance of nearly one-fifth of the tested lines indicates effective transfer of adaptive traits from M. tianschanica Vass. and confirms the suitability of this species as a donor for improving salinity tolerance in cultivated alfalfa. In respect to the testing phase, the limited testing environment which are the saline soils in the Kyzylorda Region, Kazakhstan limits our scope to a specific soil type. Future research is recommended to understand the performance and stability of these lines under other stress conditions and also in more favorable conditions. Breeding for abiotic stress tolerance is frequently associated with trade-offs in biomass accumulation or seed productivity, as adaptive traits of wild species may negatively affect yield potential. In the present study, however, the selected salt-tolerant lines maintained stable productivity of green mass and seeds under saline field conditions, suggesting a favorable balance between adaptation and agronomic performance. Similar observations have been reported in alfalfa breeding programs where wild germplasm was used to enhance stress tolerance without substantial yield penalties (Humphries et al., 2021; Selim et al., 2024). This balance is critical for the practical utilization of such material in breeding programs targeting marginal environments. The genotypes selected for their salt resistance in a hot climate in the first year of life will be additionally tested in the second year of life and used as source forms for synthetic selection to create varieties with increased adaptability to

**Table 4:** Characteristic of the selected backcrossed lines of *M. tianschanica Vass* x *M. sativa L.* by economically valuable traits

| Selection number | Corolla coloration | Bush type | Plant height, cm | Spareness, % | Productivity, gr/r.m. | | | Leafiness, % |
|---|---|---|---|---|---|---|---|---|
| | | | | | Green mass | Dry mass | Seeds | |
| Backcrosses selected from original plants with a blue-green corolla | | | | | | | | |
| 8 | violet | 2 | 55.2 | 12.4 | 465±9.4 | 63 | 3.1±0.06 | 32 |
| 14 | blue | 1 | 62.5 | 15.3 | 361±6.2 | 71 | 4.2±0.08 | 39 |
| 16 | violet | 3 | 62.0 | 16.0 | 508±5.9 | 61 | 5.0±0.04 | 37 |
| 18 | lilac | 3 | 57.0 | 16.0 | 489±8.2 | 59 | 5.3±0.05 | 33 |
| 19 | blue | 2 | 58.0 | 20.2 | 500±10.0 | 60 | 2.1±0.04 | 38 |
| 23 | violet | 2 | 50.1 | 13.0 | 348±7.5 | 33.1 | 3.8±0.07 | 27 |
| 24 | lilac | 1 | 53.2 | 12.6 | 446±9.3 | 38.6 | 4.3±0.08 | 29 |
| 25 | blue | 2 | 53.7 | 8.5 | 502±10.1 | 39.8 | 3.9±0.08 | 29 |
| 26 | violet | 3 | 62.1 | 8.2 | 600±11.3 | 61.2 | 5.0±0.09 | 36 |
| 28 | violet | 1 | 38.5 | 13.1 | 500±9.5 | 57.9 | 4.2±0.08 | 37 |
| 32 | blue | 2 | 58.6 | 9.2 | 482±9.6 | 65.7 | 4.6±0.09 | 38 |
| 33 | violet | 3 | 56.2 | 9.3 | 403±8.0 | 63.2 | 3.9±0.07 | 39 |
| 43 | lilac | 1 | 56.2 | | 306±6.0 | 49.2 | 2.5±0.06 | 37 |
| 67 | blue | 2 | 63.2 | 10.3 | 508±9.8 | 59.3 | 3.6±0.07 | 48 |
| 68 | violet | 3 | 73.3 | 6.4 | 635±11.4 | 63.6 | 4.9±0.0.5 | 49 |
| 71 | blue | 3 | 73.2 | 6.3 | 649±12.6 | 68.3 | 4.7±0.06 | 46 |
| Backcrosses selected from original plants with a dirty yellow corolla | | | | | | | | |
| 1 | lilac | 2 | 60.3 | 20.1 | 340±6.7 | 60 | 3.1±0.06 | 36 |
| 15 | violet | 2 | 60.0 | 16.6 | 506±9.8 | 62 | 4.8±0.08 | 37 |
| 17 | lilac | 2 | 61.0 | 16.0 | 507±10.4 | 61 | 3.7±0.06 | 37 |
| 22 | blue | 2 | 55.6 | 17.3 | 461±9.6 | 39 | 4.0±0.05 | 36 |
| 27 | lilac | 1 | 57.6 | 14.4 | 501±10.4 | 57.2 | 3.6±0.04 | 37 |
| 35 | lilac | 3 | 57.8 | 15.4 | 465±9.4 | 56.5 | 4.6±0.05 | 37 |
| 39 | blue | | 68.2 | 11.2 | 562±11.0 | 56.5 | 4.3±0.08 | 39 |
| 69 | violet | 2 | 58.6 | 8.8 | 672±12.4 | 65.3 | 4.9±0.09 | 43 |
| 72 | lilac | 1 | 66.8 | 9.0 | 643±11.8 | 65.3 | 3.9±0.07 | 46 |
| 80 | blue | 1 | 67.6 | 10.3 | 682±12.6 | 65.3 | 5.7±0.08 | 43 |

Note: 1 — erect; 2 — sprawling; 3 — recumbent.

abiotic factors (Dulambayeva et al., 2023; Ualiyeva et al., 2025). Overall, the results demonstrate the effectiveness of a pre-breeding strategy combining interspecific hybridization, systematic backcrossing, phenotypic marker-based selection, and field evaluation under natural salinity stress. The selected lines do not represent finished cultivars but constitute valuable source material for synthetic breeding aimed at developing alfalfa varieties adapted to saline and arid regions. This approach is especially relevant for regions experiencing increasing soil salinization and water scarcity, where conventional high-yielding varieties fail to realize their productive potential (Ualiyeva et al., 2025).

## Conclusion

The selected *M. tianschanica Vass*. plants were included in the selection program as a donor for drought and salt tolerance in backcrosses with *M. sativa L.* The $BC_1$–$BC_3$ backcross generations showed changes in bean setting, corolla color, pod turnover (by the number of revolutions), bush type, and seed hardness in the direction of the blue corolla color characteristic of *M. sativa L.* By applying backcrossing to displace undesirable genes from the wild species in hybrids and selecting blue-flowered individuals with erect bushes, spiraled pods, and low seed hardness, 27 items were selected for the synthetic selection of alfalfa as source material. To confirm the actual degree of introgression between *M. tianschanica Vass*. and *M. sativa L.,* future studies should go beyond phenotypic marker traits and incorporate contemporary molecular techniques. Applying SSR, SNP, GBS, or whole-genome resequencing techniques would make it possible to precisely identify donor genomic segments that have been preserved over backcross generations and verify whether introgressed chromosomal regions are linked to adaptive traits, especially salt and drought tolerance. By distinguishing genuine carriers of adaptive loci from individuals who display advantageous phenotypes due to environmentally induced or polygenic effects, such molecular verification will allow for a more precise evaluation of the stability of transferred alleles, identify possible cryptic introgression, and aid in the improvement of the selection scheme. Future research should concentrate on establishing trait-marker associations for important adaptive and agronomic traits found in this study in addition to molecular confirmation of introgression. The efficiency of breeding programs based on wild relatives would be greatly increased by identifying QTLs or candidate genes underlying salt resistance, regrowth capacity, decreased seed hardness, and increased biomass productivity. Furthermore, a more thorough assessment of trait stability and genotype-environment interactions would be possible by extending multi-location trials of the chosen BC lines across various stress gradients. Once genomic data are available, incorporating genomic selection models could speed up the creation of synthetic alfalfa varieties with better adaptability.

## DECLARATIONS

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**Data Availability:** Data will be available at request.

**Ethics Statement:** This study did not involve human participants or animals. All field and breeding experiments were conducted on plant materials in accordance with institutional and national guidelines for agricultural research.

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**Generative AI Statement:** The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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