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DEVELOPMENT OF MUTANT FORMS OF *AGRIOPHYLLUM SQUARROSUM* (L.)
MOQ. VIA EMS MUTAGENESIS FOR DOMESTICATION IN ARID
ENVIRONMENTS

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Abstract

Agriophyllum squarrosum (L.) Moq. is a drought-tolerant psammophytic species with high potential for domestication in arid regions; however, its agricultural use is limited by unfavorable traits such as small seed size and unstable productivity. This study aimed to develop an effective method for inducing genetic variability using ethyl methanesulfonate (EMS) and to evaluate resulting mutant forms. Seeds were treated with 0.3% EMS for 18 h, followed by washing, drying, and cultivation under controlled and field conditions. Phenotypic assessment was conducted in the M₂ generation. EMS treatment resulted in viable mutant populations with increased phenotypic diversity. Compared with the wild type, mutant plants exhibited significantly greater bush width and pronounced improvements in seed traits. The most significant differences were observed for seed width, seed length, and seed circularity. Seed length increased from 2.4 to 2.8 mm, seed width from 1.8 to 2.0 mm, and thousand seed weight from 1.83 to 2.17 g. Morphological variation was also observed in plant architecture and leaf traits. The results confirm that EMS mutagenesis is an effective tool for expanding genetic diversity in *A. squarrosum*. The identified mutant forms represent promising material for further selection and support the potential domestication of this species for cultivation in arid environments.

Keywords: EMS mutagenesis, induced variability, seed traits, desert plants, sand rice, breeding.

ҚҰРҒАҚ АЙМАҚТАРҒА ЕНГІЗУ ҮШІН *AGRIOPHYLLUM SQUARROSUM* (L.)
МОQ. МУТАНТТЫ ФОРМАЛАРЫН EMS-МУТАГЕНЕЗ НЕГІЗІНДЕ ЖАСАУ

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Аңдатпа

Agriophyllum squarrosum (L.) Moq. – қуаңшылыққа төзімді псаммофитті түр, аридті аймақтарда domestikациялау үшін жоғары әлеуетке ие; алайда оның ауыл шаруашылығында пайдаланылуы тұқымдарының ұсақ болуы және өнімділігінің тұрақсыздығы сияқты қолайсыз белгілермен шектеледі. Зерттеудің мақсаты этилметансульфонат (EMS) қолдану арқылы генетикалық өзгергіштікті индукциялаудың тиімді әдісін әзірлеу және алынған мутантты формаларды бағалау болды. Тұқымдар 0,3% EMS ерітіндісімен 18 сағат бойы өңделіп, кейін жуылып, кептіріліп, бақыланатын және далалық жағдайларда өсірілді. Фенотиптік бағалау М₂ ұрпағында жүргізілді. EMS өңдеуі фенотиптік әртүрлілігі жоғары өміршең мутантты популяциялардың қалыптасуына әкелді. Жабайы типтегі өсімдіктермен салыстырғанда, мутанттар бұтаның айтарлықтай үлкен енімен және тұқым өлшемдерінің айқын

жаксаруымен ерекшеленді. Ең айқын айырмашылықтар тұқымның ені, ұзындығы және жұмырлығы бойынша байқалды. Тұқым ұзындығы 2,4-тен 2,8 мм-ге дейін, ені – 1,8-ден 2,0 мм-ге дейін, ал 1000 тұқымның массасы – 1,83-тен 2,17 г-ға дейін артты. . Сонымен қатар өсімдік архитектурасы мен жапырақ белгілері бойынша морфологиялық өзгергіштік байқалды. Алынған нәтижелер EMS-мутагенездің *A. squarrosus* түрінде генетикалық әртүрлілікті кеңейтудің тиімді құралы екенін растайды. Анықталған мутантты формалар әрі қарай селекция үшін перспективалы бастапқы материал болып табылады және бұл түрді аридті жағдайларда өсіру мақсатында доместикациялау әлеуетін көрсетеді.

Кілт сөздер: EMS-мутагенез, индукцияланған өзгергіштік, тұқым белгілері, шөл өсімдіктері, құмаршық, селекция.

СОЗДАНИЕ МУТАНТНЫХ ФОРМ *AGRIOPHYLLUM SQUARROSUM* (L.) МОQ. С ИСПОЛЬЗОВАНИЕМ EMS-МУТАГЕНЕЗА ДЛЯ ВНЕДРЕНИЯ В ЗАСУШЛИВЫХ РЕГИОНАХ

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Аннотация

Agriophyllum squarrosus (L.) Moq. – засухоустойчивый псаммофитный вид с высоким потенциалом доместикации в аридных регионах; однако его сельскохозяйственное использование ограничено неблагоприятными признаками, такими как малый размер семян и нестабильная семенная продуктивность. Целью настоящего исследования являлась разработка эффективного метода индукции генетической изменчивости с использованием этилметансульфоната (EMS), а также оценка полученных мутантных форм. Семена обрабатывали 0,3% раствором EMS в течение 18 ч с последующей промывкой, сушкой и выращиванием в контролируемых и полевых условиях. Фенотипическую оценку проводили во втором поколении (M2). Обработка EMS привела к формированию жизнеспособных мутантных популяций с повышенным уровнем фенотипического разнообразия. По сравнению с растениями дикого типа мутанты отличались существенно большей шириной куста и заметным улучшением размеров семян. Наиболее выраженные различия были отмечены по ширине, длине и округлости семян. Длина семян возросла с 2,4 до 2,8 мм, ширина – с 1,8 до 2,0 мм, а масса 1000 семян – с 1,83 до 2,17 г. Также была выявлена морфологическая вариабельность по признакам архитектуры растений и листьев. Полученные результаты подтверждают, что EMS-мутагенез является эффективным инструментом расширения генетического разнообразия *A. squarrosus*. Выделенные мутантные формы представляют собой перспективный исходный материал для дальнейшей селекции и подтверждают потенциал доместикации данного вида для возделывания в аридных условиях.

Ключевые слова: EMS-мутагенез, индуцированная изменчивость, признаки семян, пустынные растения, кумарчик, селекция.

Introduction

Agriophyllum squarrosus (L.) Moq. is an annual psammophytic plant widely distributed across arid and semi-arid regions of Central Asia [1]. In Kazakhstan, it is found across the sandy desert and steppe zones. It is one of four species of the *Agriophyllum* genus present in the country [2]. Its exceptional adaptation to extreme environmental conditions, including drought, high temperatures, and nutrient-poor sandy soils, make this species increasingly recognized as a promising candidate for domestication in marginal lands [3]. All species of the *Agriophyllum* genus in their young form are pasture forage plants for sheep and camels. Seeds of *A. squarrosus* (also known as “sand rice”) have historically been used as food by local shepherds. The seeds were available alternative to cereals during drought seasons. They contain

high levels of proteins, lipids, and carbohydrates, comparable to major crops [1, 4]. Also, *A. squarrosus* plays an important ecological role in desert ecosystems, contributing to soil stabilization and ecosystem restoration [5]. The sand rice's contribution to combating desertification is no less significant. Its long and extensive root system can reliably anchor sand blown by strong desert winds, thus preventing erosion.

Despite its high potential, the domestication of *A. squarrosus* is constrained by several unfavorable agronomic traits. Mostly these are strong seed shattering, asynchronous maturation, small seed size, and unstable productivity under fluctuating environmental conditions [6]. These limitations significantly hinder its transformation into a cultivated crop. Therefore, the generation of novel genetic variability is a key prerequisite for overcoming these barriers and accelerating breeding progress.

Induced mutagenesis is a commonly used approach for the induction of genetic diversity in plants. Chemical mutagens, such as ethyl methanesulfonate (EMS), N-methyl-N-nitrosourea (NMU), and sodium azide (NaN_3), are well-known to cause nucleotide substitutions, deletions, and chromosomal rearrangements [7]. They have been commonly used for the induction of new allelic variants that may lead to beneficial phenotypic changes [8]. Among these methods, chemical mutagenesis using ethyl EMS is one of the most widely applied techniques. EMS primarily induces point mutations through alkylation of guanine bases, leading to G/C to A/T transitions, which can result in altered gene function and novel phenotypic traits [9]. Compared to spontaneous mutations, EMS mutagenesis significantly increases mutation frequency and allows the generation of large mutant populations suitable for selection [10]. EMS mutagenesis has proven effective across a wide range of crop species. Work in *Oryza sativa* has shown that EMS-treated populations can yield mutants with meaningfully improved agronomic characteristics [11]. A similar picture emerges from *Solanum melongena*, where EMS treatment generated substantial genetic and morphological diversity, from which lines carrying desirable traits could be reliably selected [9]. Taken together, these cases illustrate how broadly useful EMS can be, both as a crop improvement strategy and as a tool for dissecting gene function.

Attention has more recently turned to *A. squarrosus*, and early results are encouraging. EMS treatment has been shown to substantially broaden phenotypic variability in sand rice, with particularly clear effects on seed size and the timing of seedling emergence [12]. Compared to wild-type material, mutant populations spanned a considerably wider range of variation, and certain selected lines stood out for larger seeds, more uniform emergence, and stronger seedling vigor. Beyond these quantitative shifts, EMS mutagenesis has also enabled the recovery of specific morphological variants – trichome-deficient mutants being a notable example – which have in turn opened a route to identifying genes underlying adaptive traits [13]. That is why chemical mutagenesis is a workable and productive approach for both the domestication and broader genetic improvement of this species.

Induced mutations become heritable and stable in subsequent generations, particularly from the M_2 generation onward, allowing reliable selection of desirable phenotypes [14]. The integration of mutagenesis with selection provides an effective strategy for developing improved plant forms within a relatively short time frame.

Because of the ecological resilience and nutritional value of *A. squarrosus*, the expansion of its genetic variability through EMS mutagenesis is a promising approach for its domestication and utilization in arid agriculture. However, there remains a need for optimized and reproducible protocols that ensure a balance between mutation efficiency and seed viability.

The aim of this study was to develop an effective method for inducing genetic variability in *A. squarrosus* using EMS mutagenesis and to evaluate the resulting mutant populations for

key morphological and productivity-related traits. The proposed approach is expected to facilitate the development of improved forms suitable for cultivation in arid environments and contribute to the diversification of crop resources.

Research materials

Mature seeds of *A. squarrosus* were collected in September 2024. They were collected in the sandy desert near Birlik village located in Almaty region (44.6985° N, 76.7309° E). Before the treatment, seeds were manually cleaned from impurities and insects. Then they were visually inspected to select uniform, healthy, and fully developed seeds.

Selected clean seeds were pre-soaked in distilled water and kept at room temperature for 5 h to ensure uniform hydration. 0.3% EMS (Sigma-Aldrich) solution prepared in 0.1 M phosphate buffer was used as a chemical mutagen. The mutagen solution was freshly prepared immediately before use. Pre-soaked in water seeds were left in the EMS solution and incubated at room temperature for 18 h with gentle mixing to ensure uniform exposure. Following treatment, seeds were thoroughly rinsed with distilled water for 90 min to remove residual mutagen and stop its activity. After washing, seeds were placed on filter paper and air-dried at room temperature to their initial moisture content. Treated seeds were then sown in 1 m³ pots with a sand from Birlik village to simulate natural growing conditions. Germination and plant growth were carried out under following conditions: temperature of 25-35°C, relative humidity of 30-40%, and light intensity of 80000-120000 lux. A total of 150 seeds were treated with EMS. Of these, 51 seeds germinated in the M₁ generation, corresponding to a germination rate of 34%.

The seeds of M₁ generation were grown in greenhouse of Institute of plant biology and biotechnology. Randomly selected seeds harvested from M₁ plants were used to establish an M₂ population consisting of 15 individuals. Since all available M₂ plants were included in the study, no additional sampling was performed. The relatively small M₂ population size (n = 15) should be considered a limitation of the present study, as it may not fully capture the spectrum of EMS-induced mutations. All M₂ plants were included in the phenotypic evaluation, where recessive EMS-induced mutations can become phenotypically expressed following segregation. M₂ plants along with wild type plants were grown in test field in Birlik village in April 2025. Plants were assessed based on key morphological and productivity-related traits, including plant height, bush width, seed size, and thousand seed weight. Seed length, seed width, and seed circularity were determined using a MARVIN digital seed analyzer (MARViTECH GmbH, Germany). Seed circularity represents a software-derived shape parameter calculated automatically by the MARVIN image analysis system and used to characterize seed geometry. Fifteen plants per wild and mutant forms were assessed. A sample size of fifteen plants per group is commonly considered sufficient for preliminary mutagenesis studies aimed at detecting major phenotypic effects and identifying promising mutant lines for further evaluation. Statistical analyses were performed using R software v. 4.4.0. Differences in trait values between wild and mutant forms were evaluated using Student's t-test. Prior to analysis, data normality was assessed using the Shapiro–Wilk test.

Results and Discussion

The application of EMS mutagenesis to seeds of *A. squarrosus* resulted in the successful generation of a viable mutant population. They were characterized by visible phenotypic variability. The treated seeds demonstrated good germination rates, indicating that the selected EMS concentration and exposure duration provided a balance between mutagenic efficiency and seed viability. This is consistent with previous reports highlighting the importance of

optimizing EMS dosage to avoid excessive lethality while maintaining mutation frequency [12].

Early developmental stages revealed no critical abnormalities that would compromise plant survival. As shown in Figure 1A–C, germination occurred uniformly, and seedlings developed normally under controlled conditions.



Figure 1. Developmental stages of *Agriophyllum squarrosum* following EMS treatment (M_1). (A) Germination of treated seeds under controlled conditions; (B) Early seedling emergence in sandy substrate; (C) Seedling establishment under artificial lighting; (D) Vegetative growth stage showing plant architecture and leaf development; (E) Close-up of stem and leaf morphology illustrating trichomes and structural features; (F) Close-up of flowers.

Slight variation in germination dynamics was observed, suggesting the presence of mutations affecting early growth processes. In Figure 1D-F, plants at later vegetative stages exhibited differences in leaf morphology, branching patterns, and plant architecture, reflecting the induction of genetic variability.

A key outcome of EMS treatment was the expansion of phenotypic diversity compared to the original population. Morphological assessment of the M₂ generation revealed significant variation in plant height and bush width (Figure 2).



Figure 2. Wild type plant (A) and mutants (M₂) (B) of *Agriophyllum squarrosum* in the experimental plot.

Growing the two forms side by side in field conditions revealed the morphological differences between the wild-type and mutant form of *A. squarrosum*. The wild type (Figure 2A) had an open, loosely branched structure. Its internodes were elongated, lateral shoots were sparsely arranged, and the stems were slender than in mutants. The mutant form (Figure 2B) looked strikingly different. Its growth habit was markedly compact and sturdy, with shorter internodes and a denser arrangement of branches. The inflorescences were clustered together into tight spike-like structures – larger than those of the wild type. The mutant also developed thicker stems, and its reproductive organs were distributed more evenly and regularly along the shoots – a feature that, from a breeding standpoint, may be of considerable practical interest.

Quantitative analysis (Table 1) demonstrated that mutant lines exhibited slightly increased plant height (43.8 ± 3.5 cm) compared to the wild population (41.5 ± 4.9 cm), as well as a wider bush architecture (23.6 ± 1.7 cm vs. 21.7 ± 2.7 cm). The greatest significant difference between wild and mutant forms was observed for seed length, width, and circularity

(P-values from 7.54×10^{-6} to 6.00×10^{-13}). These traits may contribute to improved potential yield under cultivation conditions.

Table 1. Comparative analysis of morphological and productivity-related traits wild and mutant lines of *Agriophyllum squarrosum* based on Student's t-test.

Form	Plant height, cm	Bush width, cm	Seed width, mm	Seed length, mm	Seed circularity	1000 seeds weight, g
Wild	41.5 ± 4.9	21.7 ± 2.7	1.8 ± 0.1	2.4 ± 0.2	1.2 ± 0.08	1.83 ± 0.41
Mutant	43.8 ± 3.5	23.6 ± 1.7	2.0 ± 0.06	2.8 ± 0.2	1.6 ± 0.03	2.17 ± 0.17
P-value of the intergroup difference	0.1514	0.0302	9.07×10^{-7}	7.54×10^{-6}	6.00×10^{-13}	0.0080

Particularly visible changes were observed in seed morphology as well (Figure 3).

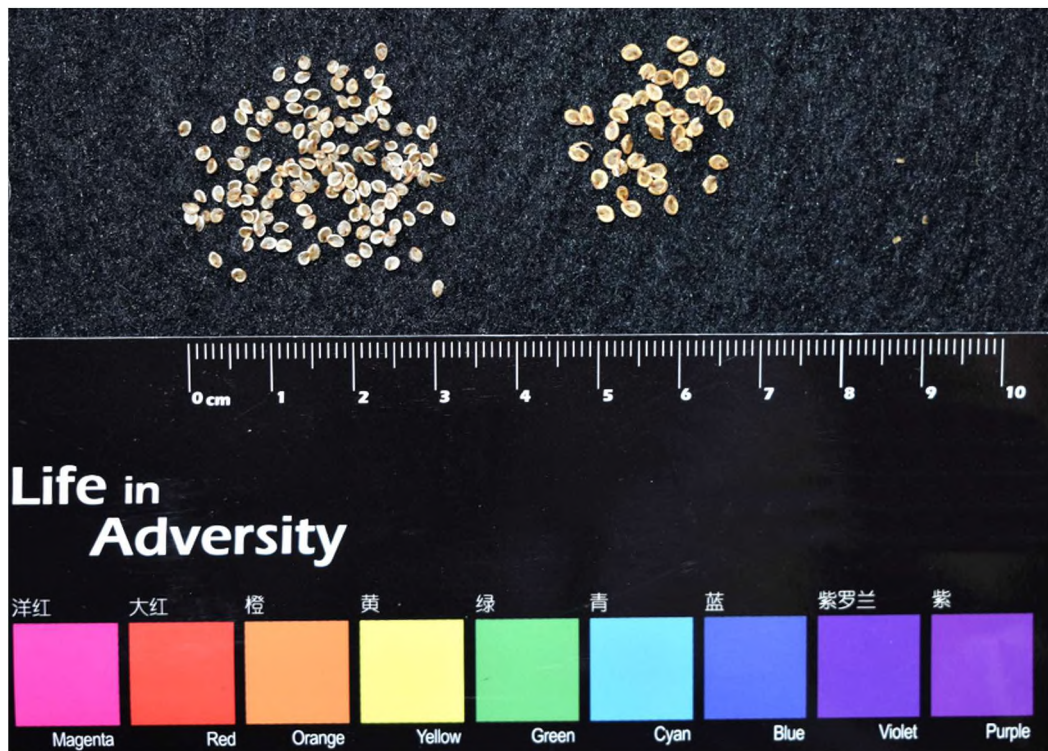


Figure 3. Comparison of seed sizes in wild forms (on the left) and mutant forms (on the right) of *Agriophyllum squarrosum*.

Mutant seeds were visually larger and more uniform than seeds from the wild population. This observation was supported by biometric measurements. Seed width increased from 1.8 ± 0.1 mm in wild-type seeds to 2.0 ± 0.06 mm in mutant forms, while seed length increased from 2.4 ± 0.2 mm to 2.8 ± 0.2 mm, respectively (Table 1). Despite that, seed circularity also increased significantly, indicating that mutant seeds became more rounded in shape in addition to being larger. The thousand seed weight increased from 1.83 ± 0.41 g in the control to 2.17 ± 0.17 g in the mutant line, representing a substantial improvement in a key agronomic trait.

The increase in seed size and weight is particularly significant in the context of domestication. Small seed size is one of the primary limitations of *A. squarrosum* as a potential crop [15, 16]. Larger seeds not only improve yield potential of mutant lines, but also facilitate sowing and increase competitiveness under field conditions of extreme deserts. Similar improvements in seed traits after EMS mutagenesis have been reported in other species [10, 12, 17]. In general, it confirms the effectiveness of this approach in modifying yield-related characteristics.

Along with the seed traits, variation in plant morphology was also detected. Differences in leaf length, width, and orientation were observed among mutant individuals suggesting alterations in developmental pathways of plant vegetative organs as well. Changes in plant architecture, including branching and stem size, may influence resistance to lodging and adaptability to cultivation practices. These findings indicate that EMS-induced mutations can affect multiple trait categories simultaneously [12, 13], providing a broad selection base for breeding.

The observed variability was consistently expressed in the M₂ generation, indicating successful induction and phenotypic manifestation of EMS-derived mutations [13, 18]. This is a critical requirement for their use in breeding programs. However, because M₂ represents a segregating generation, stable inheritance of the observed phenotypes should be confirmed through evaluation of subsequent generations (M₃, M₄ and etc.). The ability to identify and select desirable phenotypes at this stage significantly accelerates the development of improved lines compared to conventional selection from natural populations. From a practical perspective, the developed mutagenesis protocol proved to be efficient and reproducible. The use of a single EMS treatment step simplifies the procedure and reduces labor and resource requirements. At the same time, the method generates sufficient variability to enable effective selection. Compared to more complex mutagenesis systems involving multiple treatments or additional genetic manipulations, this approach is well-suited for routine use in breeding and research programs. A limitation of the present study is the absence of a buffer-treated control group. Although the observed phenotypic variation is consistent with previously reported EMS-induced effects in *A. squarrosum* and other species, a contribution of seed soaking-related physiological responses cannot be fully excluded. The present study was based exclusively on phenotypic evaluation and did not include molecular verification of EMS-induced mutations. Therefore, the observed phenotypic variation cannot be unequivocally attributed to specific DNA sequence changes. Although EMS predominantly induces point mutations, contributions of epigenetic modifications or other non-genetic factors cannot be completely excluded. Future work will focus on molecular characterization of selected mutant lines using genotyping and/or sequencing-based approaches to validate the genetic basis of the observed phenotypes.

However, the results demonstrated that EMS-induced mutagenesis is a powerful tool for expanding phenotypic diversity of *A. squarrosum*. The identified mutant forms with improved seed size, plant architecture, and potential increased productivity traits represent valuable material for further domestication and breeding. Due to the ecological resilience of this species, such improvements may contribute to the development of new crops adapted to arid and semi-arid environments in Kazakhstan and other Central Asian countries.

Conclusions

The results of this study demonstrated that EMS-induced mutagenesis works well as a strategy for generating phenotypic variation expressed in the M₂ generation in *A. squarrosum*. The treatment conditions we settled on – 0.3% EMS applied for 18 hours – struck a workable balance between inducing mutations and maintaining acceptable seed viability, ultimately

yielding a mutant population expressing stable phenotypes within the evaluated M₂ generation. By the M₂ generation, meaningful improvements had emerged across several agronomically relevant traits: bush width, seed width, seed length, seed circularity, and thousand seed weight all showed measurable gains. The increases in seed size and weight are perhaps the most practically significant of these, as small seed size has long been considered one of the main obstacles standing between this species and successful domestication. Beyond seed traits, the mutagenesis also produced variation in plant architecture and seed uniformity, which broadens the pool of characteristics available for selection. The expression of these traits in the M₂ generation suggests that EMS treatment successfully generated novel phenotypic variation. However, confirmation of stable inheritance will require evaluation of subsequent generations. On the whole, the protocol described here is straightforward, reproducible, and yields material of real breeding value – and the findings add further weight to the idea that *A. squarrosum* deserves serious consideration as a candidate crop for dryland and arid agricultural systems. Future studies will involve larger populations, multi-environment trials, and evaluation of additional agronomic traits to validate the stability and breeding value of the selected mutant lines.

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Conflict of interests

The authors declare no conflict of interest.

Authors contribution

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Y.A. Genievskaya – investigation, data curation, writing-original draft preparation, writing-review and editing; S.A. Abugalieva – writing-review and editing; Y.K. Turuspekov – conceptualization, writing-review and editing; A.K. Zatybekov – data curation, methodology, writing-review and editing, funding acquisition, project administration.

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