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In Vitro Micropropagation of Selected Halophytic Species from Family the *Chenopodiaceae (Amaranthaceae)*

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Abstract: This study focuses on the in vitro micropropagation of selected halophytic species belonging to the *Chenopodiaceae (Amaranthaceae*) family, collected from ecologically diverse and arid regions of Uzbekistan, including Sirdarya, Bukhara, and the drained Aral Sea zone (Muynak district). The primary objective was to establish sterile cultures, optimize the growth conditions, and evaluate the regenerative capacity and biomass accumulation of these salt-tolerant plants under controlled laboratory conditions.Sterile explants were obtained from seedlings germinated on Murashige and Skoog (MS) media supplemented with kinetin, BAP, or without growth regulators. Under these conditions, active germination was observed as early as days 3–6 of cultivation. The application of auxins and their combinations with kinetin, as well as 6-BAP—excluding media containing NAA - induced shoot regeneration across most annual halophyte species during both initial and subsequent subcultures. This led to a significant increase in explant proliferation and plant biomass production.For secondary subcultures of *Salsola dendroides, Salsola orientalis*, and *Salsola richteri*, Woody Plant Medium (WPM) was employed, demonstrating its suitability for maintaining and multiplying regenerants. The study highlights the effectiveness of specific plant growth regulators and media formulations in enhancing in vitro propagation efficiency.These results offer a promising foundation for the conservation, sustainable use, and possible reintroduction of halophytes into degraded saline environments, particularly the former Aral Sea bed. The established micropropagation protocols may further serve as a model for other stress-tolerant plant species with ecological and medicinal potential.

Keywords: In Vitro, Halophytes, Explant, Sterilization, Propagation.

Introduction

In the desert regions of Uzbekistan, the leading environmental stressors include an arid, sharply continental climate, high salinity levels in water and soil, pollution, and a shortage of potable water resources, as well as widespread desertification of formerly productive lands (Kamalov et al., 2001). These issues are particularly severe in the Aral Sea region, where the degradation of environmental conditions has been driven by a combination of natural and anthropogenic factors. This has led to a dramatic decline in sea levels and the frequent occurrence of dust and salt storms arising from the desiccated seabed.

Salinized soils are unsuitable for conventional agricultural crops, resulting in reduced productivity and economic losses in land management. However, certain promising halophytic species demonstrate high ecological adaptability and offer potential for the rehabilitation of saline lands and the establishment of specialized plantations. Halophytes serve as an important biological resource for agriculture, environmental restoration, and even medicinal applications (Waisel, 1972; O'Leary, 1985).

Many representatives of the *Chenopodiacea*e family are key edificators of halophytic vegetation in Uzbekistan. Modern scientific strategies for the sustainable use of plant resources in arid zones are closely linked to the development of plant biotechnology. In this context, the development of in vitro techniques for large-scale production of genetically stable microplants is of particular interest. This includes obtaining sterile and pathogen-free starting material suitable for regeneration and optimizing micropropagation conditions to promote shoot development and root system formation (Butenko, 1999).

Method

The study focused on long-vegetating halophytic species of the genera *Climacoptera, Atriplex, Suaeda, Halocnemum*, and various species of *Salsola*, collected in 2024 from the southern Aral Sea region, Republic of Karakalpakstan (Uzbekistan). To establish in vitro cultures, both seeds and seedlings were used as explants. The in vitro introduction process was divided into two main stages:

a) Sterilization (Decontamination): Plant material was treated with strong sterilizing agents to eliminate bacterial and fungal contamination and to obtain sterile explants.
b) Cultivation: The sterilized explants were cultured under standard in vitro conditions using agar-solidified nutrient media, following well-established protocols in plant biotechnology. At the end of each subculture (passage), the developmental responses of explants were recorded and analyzed (Dunaeva et al., 2017; Murashige & Skoog, 1962).

Results and Discussion

At the initial stage of disinfection, a 70% ethanol solution was applied for one minute. This was followed by the main phase of seed treatment, during which such parameters as reagent concentration and exposure time were optimized based on preliminary experiments. A variety of reagents were tested during the study, including Diacid, mercuric chloride, soap solutions, ethanol at different concentrations (70%, 75%, and 96%), and various dilutions of bleach solution (ratios of bleach to water: 1:10 and 1:20).

Significant results were achieved with a 0.001% solution of thimerosal applied for 30 minutes, treatment with 70% ethanol for 15–30 minutes, and triple rinsing with sterile distilled water after each treatment. This approach significantly increased the proportion of viable seeds. After the aseptic treatment phase, the solution was removed, and the seeds were carefully rinsed with sterile distilled water, further optimizing the percentage of seeds that retained viability (Khalbekova, 2023, 2024). Subsequently, the treated material was transferred onto a specified nutrient medium. Analysis of experimental results revealed that the proportion of plants free from endogenous microbial contamination varied depending on the species. Some species exhibited minimal internal microbial presence, resulting in a high percentage of sterile plants-ranging from 60% to 90%.

In this context, detailed optimization of growth regulator concentrations was conducted for in vitro cultures of plant species such as *Climacoptera, Atriplex, Suaeda, Halocnemum*, and *Salsola*. It is important to highlight that members of the Chenopodiaceae family showed a positive response to the introduction of growth stimulators. This effect was evident at the early stages of the cultivation process, significantly reducing the time between sowing and germination.

The emergence of the first seedlings in the studied species was observed within 7–15 days after sowing. After 28 days, the seedlings were transferred to fresh nutrient media. Cultivation of microcuttings on Murashige and Skoog (MS) medium led to the development of one to two shoots with elongated internodes, although the root systems were only weakly developed. Within 5–10 days after transferring explants to hormone-free MS medium, synchronous proliferation was observed.

For the first subculture, the optimal conditions were full-strength MS; for the second, ½ MS supplemented with 0.5 mg/L 6-benzylaminopurine (BAP) and 0.3 mg/L indole-3-butyric acid (IBA). To increase the number of regenerants, the following combinations were tested: ½ MS + 0.5 mg/L BAP + 0.5 mg/L kinetin (Kin) and ½ MS + 0.5 mg/L BAP + 0.3 mg/L IBA.

The stunted growth observed in seedlings from the Muynak region is likely due to their adaptation to extreme environmental conditions, which may result in lower nutrient demand during microshoot development. For the

continued growth of microshoots, a suitable medium was ½ MS supplemented with 0.5 mg/L BAP and 0.5 mg/L Kin, where the mortality rate of regenerants ranged from 12.1% to 28.7%.

Interestingly, optimal germination of aseptic seeds and formation of primary root systems in the studied species occurred under two conditions: ½ MS supplemented with 0.5 mg/L BAP + 0.3 mg/L IBA (60%) and hormone-free MS (90%). These media significantly stimulated in vitro seedling propagation. In contrast, species such as *Halocnemum* and *Salsola* required longer periods and higher hormone doses: initial shoot emergence was observed on day 10 after sowing, while complete leaf formation occurred within the next 7–10 days.

In vitro seed germination studies revealed low germination rates on media with various concentrations of the phytohormone Kin. However, when combined with the cytokinin BAP, both seed germination and seedling development were enhanced. Rooting of the initial explants typically began after 4–5 weeks. Under experimental conditions where the concentration of cytokinins exceeded that of auxins by 5–10 times, direct shoot formation was observed.

Callus Induction

To obtain sterile material, after treatment, the plant material was washed twice in distilled water and transferred directly to a nutrient environment. The seeds were transferred to a hormone-free environment with half salts MS (29) with sucrose with the addition of 7.5 g/l agar as a gel-forming component (Table 1).

Table 1. Efficiency of different culture media for in vitro regeneration of *Chenopodiaceae* species

№	Culture medium composition	Regeneration frequency (%)	Growth characteristics	Callus formation	Shoot formation	Overall efficiency
1	1	Full MS + 2 mg/L 6-BAP + 1 mg/L IAA	40%	Slow, elongated shoots	Moderate	Weak
2	2	MS + 1 mg/L 6-BAP + 0.5 mg/L NAA	55%	Moderate growth, thin shoots	Slight	Average
3	3	½ MS + 1 mg/L 6-BAP + 0.3 mg/L IAA + 2,4-D *(optimal)*	85%	Active growth, compact shoots	Controlled	**Good**
4	4	MS + 1.0 mg/L 2,4-D	30%	Callus without shoot development	Strong	None
5	5	½ MS without growth regulators *(control)*	10%	No morphogenesis	None	None

In the course of the experiment, seeds of the studied *Salsola* species were first cultivated in vitro. Then, several rectangular segments (0.2–0.5 mm) were excised from the central part of the leaf blade that had developed from the germinated seeds. These explants were placed on Murashige and Skoog (MS) medium supplemented with 0.5, 1.0, and 2.0 mg/L of the plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D), with 10–12 leaf segments cultured per Petri dish. The cultures were incubated in darkness at 26 ± 2°C.

It was confirmed that the optimal concentration for callus induction and development from the leaves of *S. richteri and S. orientalis* was 0.5 mg/L 2,4-D when added to MS medium. The first hormonal responses of the explants were observed within one week. By the second week, soft whitish-yellow callus tissue had developed on media with MS + 0.5 mg/L 2,4-D. On media supplemented with MS + 0.5 mg/L 2,4-D + 0.3 mg/L 6-benzylaminopurine (BAP), relatively firm yellowish to light-green callus formed. Furthermore, improved callus quality was observed when the medium was supplemented with 0.5 mg/L 2,4-D + 0.3 mg/L BAP + 300 mM NaCl, which proved to be the most optimal formulation. Despite the taxonomic differences among the studied species, a shared biological characteristic in seed germination was noted. This pattern is also evident in the natural phytocenoses of these plants, which is likely attributable to their belonging to the same botanical family, *Chenopodiaceae*.

In particular, species of the genus Atriplex showed later stages of germination, with total germination rates ranging from 57% to 96%. On the other hand, species such as *Atriplex, Climacoptera, Chenopodium*, and *Suaeda*-despite morphological diversity ranging from shrubs to small trees like *Halocnemum* and *Salsola*-demonstrated a high germination capacity.

Under in vitro conditions, when using ½ MS + 0.5 mg/L 6-benzylaminopurine (BAP) + 0.3 mg/L indole-3-butyric acid (IBA) and hormone-free MS medium, seed germination efficiency ranged from 60% to 90%. It is assumed that the seeds of these species exhibit different dormancy stages, which depend not only on the species but also on the position of the seeds on the generative shoot.

When MS + 1.2 mg/L kinetin (Kin) + 0.1 mg/L IBA or ½ MS + 1 mg/L Kin + 0.1 mg/L IBA were used, all studied species showed low germination rates—from 8% to 15%. Such a combination of growth regulators had a negative effect on all halophytes: physiological processes slowed down, explant necrosis was observed, and root development was poor on media containing kinetin. Upon reducing the concentration of kinetin, an increase in seed germination was noted, reaching 10–12%, while root formation also reached 10%. When mature seeds were sown on media containing kinetin, they lost viability by the 14th day of cultivation.

For the induction of rhizogenesis in rooting experiments with all studied species of the *Chenopodiaceae* family, IBA at a concentration of 0.1 mg/L was shown to be optimal, with root induction ranging from 8% to 30%. However, increasing the IBA concentration to 0.3 mg/L improved rooting efficiency up to 97%. Nonetheless, at 0.1 mg/L IBA, roots appeared earlier and in greater numbers, making the concentration range of 0.1–0.3 mg/L IBA optimal for rhizogenesis in the studied halophytes.

It was noted that on such media, the seeds of all analyzed species demonstrated active germination rates between 30% and 60%. Interestingly, on hormone-free MS medium, the germination rate for all studied species was higher, reaching 70%–80%. After 10 days of cultivation on MS medium without hormone supplements, seed germination reached an impressive 97%. In contrast, when the same seeds were placed on media containing kinetin, germination dropped to just 12%.

Shoot Regeneration

Shoot regeneration from callus was successfully achieved by transferring callus to MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L indole-3-acetic acid (IAA). The maximum regeneration rate (60%) was recorded 21 days after the callus was transferred to the regeneration medium. The developed in vitro propagation protocol for Salsola species enables the efficient production of sterile plants, callus, and regenerated shoots. Notably, successful seed sterilization proved to be a critical step, directly influencing the viability of the resulting seedlings. The high percentage of regenerants obtained following sterilization confirms the preservation of tissue physiological activity and the successful initiation of morphogenesis.

Conclusion

Thus, as a result of the conducted studies, it was determined that seeds of the studied halophyte species from the Chenopodiaceae family, when placed on media supplemented with kinetin, as well as on media containing BAP or MS without hormones, germinated actively within 3 to 6 days of cultivation. The applied auxins and their combinations with kinetin, as well as BAP (with the exception of the medium containing NAA), induced the formation of regenerants during both the initial and subsequent subcultures in all annual halophyte species, leading to an increase in the number of explants. For the secondary subcultures of Salsola dendroides, Salsola orientalis, and Salsola richteri, the ready-made WPM medium was used.

Scientific Ethics Declaration

* The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Conflicts of Interest

* The authors declare no conflict of interest.

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