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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

DEVELOPMENT OF A BIOTECHNOLOGICAL PROPAGATION METHOD FOR THE AUTOCHTHONOUS GRAPE VARIETY "SHIRVANSHAHI"

Speciality: 2422.01 – Biotechnology (including bionanotechnologies)

Field of science: Biology

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Baku – 2026

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INTRODUCTION

Relevance of the topic and degree of development. Grapevine (*Vitis vinifera* L.) is one of the oldest cultivated plants in the world and has played an important role in the development of agriculture, culture, and the economy. Archaeological studies conducted in Azerbaijan have shown that viticulture and winemaking have a very ancient history in this region. "*Based on the studies of Academician N.I. Vavilov, Azerbaijan is considered one of the primary centers of origin of grapevine cultivation*."[1]

"Most local grapevine varieties in Azerbaijan originated from wild grapevine (Vitis vinifera L. subsp. sylvestris Gmel.) as a result of natural selection. In recent years, a number of studies have been conducted on the biodiversity of wild grapevine."[2] As a result of these investigations, more than 2,000 wild grapevine plants have been identified, and their distribution areas and phytocenotic characteristics have been described. As in many parts of the world, in Azerbaijan, under the influence of adverse factors, many valuable local, breeding-derived, and introduced grapevine varieties have already been lost, while some others are currently facing the threat of extinction. In this regard, the conservation of the grapevine gene pool, its inclusion in collections, and its transmission to future generations are of great scientific importance.

Of particular interest is the Shirvanshahi variety, one of Azerbaijan's rare indigenous technical grapevine varieties.Historically cultivated mainly in the Kurdamir district, it served as an important raw material source for the production of high-quality wines and cognac distillates. However, as a result of changes in the varietal composition of vineyards, this variety has almost completely disappeared from commercial plantations and has been preserved only as individual old vine specimens.

"For this reason, biotechnological methods, especially micropropagation, are considered an effective tool for the conservation

[1] Вавилов Н.И. Центры происхождения культурных растений // Труды по прикладной ботанике, генетике и селекции. – 1926. – Т. 16, № 2. – С. 1–248.

[2] Musayev M.K. Results of the study of genetic resources of grapes in Azerbaijan // *International Journal of Plant Breeding*. – 2015.

and mass restoration of rare genotypes."[3] This method enables the collection of grapevine genetic resources and their involvement in breeding and improvement programs, as well as the development of new varieties that are more productive, resistant to biotic and abiotic environmental stress factors, high in quality, and suitable for technological use. The conservation and large-scale cultivation of indigenous grapevine varieties should not be limited solely to the application of efficient biotechnological methods. At present, from both scientific and commercial perspectives, it is necessary to determine the genetic origin of each grapevine variety, its degree of relatedness to wild forms, and its genetic relationships with technical varieties.

Object and subject of the study. The objects of the study were the indigenous technical grapevine variety Shirvanshahi (*Vitis vinifera* L. Shirvanshahi), collected from the Kurdamir district of the Republic of Azerbaijan; wild grapevine forms (*Vitis vinifera* L. subsp. *sylvestris*) collected from areas along the Kura River; and local grapevine genotypes cultivated in zones adjacent to these areas. A total of 21 grapevine samples were used in the study.

The subject of the study included the biotechnological parameters of the *in vitro* micropropagation process of the Shirvanshahi grapevine variety, namely the component composition of the nutrient medium, the lighting regime, the rooting system, and *in vitro* adaptation conditions. In addition, the study focused on the molecular-genetic diversity, phylogenetic relationships, and genotypic characteristics of the investigated grapevine samples as determined using RAPD, ISSR, and SSR markers

Aims and objectives of the study. The aim of this study was to develop a scientific and methodological protocol for the mass restoration of the rare indigenous technical grapevine variety Shirvanshahi, whose populations have sharply declined, based on micropropagation. The study also aimed to evaluate the genetic diversity and phylogenetic relationships among the Shirvanshahi variety, closely related local grapevine samples, and wild genotypes using RAPD, ISSR, and SSR molecular markers.

[3] Bouquet A., Torregrosa L. Micropropagation of the grapevine (*Vitis* spp.) // *Micropropagation of Woody Trees and Fruits* / Eds. S.M. Jain, K. Ishii. – Dordrecht: Kluwer Academic Publishers, 2003. – P. 319–352.

To achieve this aim, the following objectives were set:

- ➢ To develop a methodology for preparing donor plant material, selecting an appropriate sterilization regime, and introducing the Shirvanshahi grapevine variety into aseptic culture for micropropagation.
- ➢ To evaluate the effects of the main components of the MS nutrient medium, including nitrogen compounds, phosphorus level, and sucrose concentration, on the *in vitro* morphogenesis and vegetative development of Shirvanshahi microclones.
- ➢ To determine the effects of LED light intensity and spectral composition on the *in vitro* morphogenesis of the Shirvanshahi grapevine variety and to select the optimal light regime.
- ➢ To induce *in vitro* root formation and ensure directed development of the root system through the application of a three-component gradient nutrient medium rooting complex.
- ➢ To determine the optimal substrate variants for the adaptation of rooted microplants under *in vitro* conditions and to develop a stepwise adaptation scheme.
- ➢ To evaluate the genetic diversity among the Shirvanshahi variety, local grapevine samples, and wild *Vitis vinifera* subsp. *sylvestris* forms using RAPD, ISSR, and SSR molecular markers.
- ➢ To construct dendrograms based on the activity of different marker systems and to determine the phylogenetic relationships and genetic grouping patterns of the investigated samples using PCA and PCoA results.

Research methods. Research methods. The theoretical basis of the study was formed through an in-depth and comprehensive review of modern scientific literature. Biotechnological and molecular biological methods were widely applied in the experimental work carried out within the framework of the dissertation. All obtained results were processed and analyzed using appropriate statistical methods.

The provisions of the dissertation presented for defense:

- ➢ An *in vitro* micropropagation system for the indigenous Shirvanshahi grapevine variety has been developed, ensuring the production of viable plants and their successful adaptation under *in*

vitro conditions.

- The regulation of *in vitro* morphogenesis and development of the Shirvanshahi grapevine variety is determined by photobiotechnological factors, including the spectral composition and intensity of LED lighting, as well as the trophic components of the nutrient medium.
- A three-component gradient nutrient medium rooting complex has been developed, which ensures the formation of a functional root system in the Shirvanshahi grapevine variety suitable for *in vitro* adaptation.
- Molecular-genetic research based on RAPD, ISSR, and SSR marker analyses reveals the presence of phylogenetic relationships between the Shirvanshahi variety and local cultivated and wild grapevine genotypes.

Scientific novelty of the research: This dissertation is the first study devoted to the development of an integrated approach based on the application of modern biotechnological and molecular-genetic methods for the conservation, study, and mass restoration of the indigenous Shirvanshahi grapevine variety.

- For the first time, a comprehensive biotechnological propagation scheme covering donor material preparation, *in vitro* introduction, micropropagation, rooting, and *in vitro* adaptation stages was developed and validated for the Shirvanshahi indigenous grapevine variety.
- In order to establish a commercial propagation protocol, the optimal cultivation parameters for *in vitro* morphogenesis of the Shirvanshahi variety were determined: sucrose concentration of 30–40 g/L, a balanced ratio of NH_4NO_3 and KNO_3, as well as the regulation of KH_2PO_4 levels, exerted a complex effect on the vegetative development of microclones.
- The effects of the spectral composition and intensity of LED lighting on *in vitro* developmental parameters were systematically evaluated. A light regime consisting of 70% red and 30% blue spectra, with an irradiance of approximately 46 $\mu mol \cdot m^{-2} \cdot s^{-1}$, was identified as optimal for morphogenesis.
- For the first time, a three-component gradient rooting complex of

the nutrient medium was applied, and variant B medium, enriched with a combination of IAA and IBA under a reduced nitrogen background, was proven to be more effective in terms of rooting, lateral root formation, and root development.

- For the first time, the genetic diversity and phylogenetic relationships of the Shirvanshahi variety with local and wild genotypes were comprehensively assessed using RAPD, ISSR, and SSR markers. The integration of molecular and biotechnological approaches was presented as a new methodological framework.
- Using genotypes such as VVSH5 and VS3 as examples, the existence of high polymorphism and structural diversity within the local and wild grapevine gene pool was demonstrated.
- The obtained results provided a scientific basis for the inclusion of Shirvanshahi and other local varieties in breeding programs and large-scale restoration activities, as well as for increasing the efficiency of gene pool utilization.
- For the first time, the genetic diversity of the Shirvanshahi variety was assessed in populations distributed within its natural range and in adjacent regions.
- The results of the dissertation provide scientifically grounded methods that can be applied in practical breeding and viticulture.

Theoretical and practical significance of the study. **Theoretical and practical significance of the study.**

For the first time, a comprehensive and integrative scientific approach has been developed for the processes of morphogenesis, micropropagation, rooting, and *in vitro* adaptation in the *in vitro* culture of Shirvanshahi, one of the indigenous technical grapevine varieties. The effects of the main components of the nutrient medium, including sucrose, nitrogen, and phosphorus, as well as the spectral composition and intensity of LED lighting, on morphogenetic responses were systematically analyzed, and an optimal development model was determined.

Genetic differentiation of *Vitis vinifera* and *V. vinifera* subsp. *sylvestris* populations was determined based on RAPD, ISSR, and SSR markers. It was shown that the multimarker approach enables high-precision assessment of genetic polymorphism. Higher genetic diversity was detected in wild populations, confirming the narrowing of the genetic

base during plant domestication.

For the first time, the autochthonous origin of the Shirvanshahi variety was substantiated at the molecular level, and its high genetic similarity with wild forms from the Kura River basin was determined. The mass restoration of the Shirvanshahi variety is of strategic importance for reviving a unique brand such as "Kurdamir" dessert wine.

Publication, approval and application of the dissertation. Based on the materials of the dissertation, 19 scientific works were published in local and foreign publications, including 6 articles and 13 theses.

The main provisions of the dissertation were presented at various republican and international scientific events in different years, including the international conference dedicated to the 80th anniversary of the Institute of Botany (Baku, 2016), the International Scientific-Practical Conference on "Actual Problems of Modern Natural Sciences" (Ganja, 2017), the scientific-practical conference dedicated to the 90th anniversary of Academician Jalal Aliyev (Baku, 2018), the 1st Republican Student Scientific Conference (Baku, 2019), the 2nd International Congress of Applied Sciences (Baku, 2021), the 11th International Conference "Achievements and Challenges in Biology" (Baku, 2022), the 26th Republican Scientific Conference of Doctoral Students (Baku, 2023), the Symposium on Eurasian Biodiversity – SEAB (Erzurum, 2024), the 11th International Scientific-Practical Conference (Antwerp–Brussels, 2025), and the workshop "Temperature and Light Responses in Plants" (Agadir, 2025).

Organization where the dissertation work was carried out. The dissertation work was carried out at the Biotechnology and Bioengineering Laboratory of the Institute of Molecular Biology of the Ministry of Science and Education of the Republic of Azerbaijan and at the Department of Molecular Biology and Genetics, Faculty of Art and Sciences, Istanbul Yeni Yüzyıl University, Istanbul, Türkey.

Structure and scope of the dissertation The dissertation consists of four chapters, a final summary, conclusions, recommendations, a list of references, and a list of abbreviations. In terms of structure, the introduction comprises 15,637 characters, the first chapter 62,931 characters, the second chapter 25,883 characters, the third chapter 71,792 characters, the fourth chapter 21,714 characters, the final summary and

conclusions 8,015 characters, and the list of 286 references 51,365 characters. The dissertation consists of 167 pages of computer-typed text, with a total volume of 264,212 characters.

CHAPTER I
LITERATURE REVIEW

In this section, a comprehensive literature review was conducted on the concept of plant micropropagation, the methods and stages of micropropagation, the biology and taxonomy of grapevine as a cultivation object, and the factors affecting the efficiency of micropropagation in grapevine plants. At the same time, based on literature sources, the topics of molecular markers and phylogenetic studies in viticulture, the role of molecular markers, modern phylogenetic approaches and methodologies, the genetic relationships of local and wild grapevine populations, as well as the scientific and practical significance of molecular phylogenetic studies were reviewed

CHAPTER II
RESEARCH MATERIAL AND METHODS

The Shirvanshahi grape variety (Vitis vinifera L.) collected from the village of Chohranli, Kurdamir region of the Republic of Azerbaijan, was selected as the object of the study. It is considered a valuable source of raw materials for the production of high-quality Cahors-type dessert wines. The biological material used in research was maintained as both *in vivo* and *in vitro* collections in the laboratory of Biotechnology and Bioengineering of the Institute of Molecular Biology of the State University of Technology of the Republic of Azerbaijan. 21 grape samples collected from different regions of Azerbaijan were used for phylogenetic analyses; 16 of them belong to the subspecies *V. vinifera* subsp. *vinifera*, and 5 to the subspecies *V. vinifera* subsp. *sylvestris.*

Preparation of initial plant material for microclonal propagation. 1-2 axillary shoots were used as initial explants. For *in vitro* cultivation, the explants were sterilized and maintained in distilled water for 1-2 weeks in an artificial climate conditions at 20-22°C, then

stimulated in indolyl-butyric acid (IBA) for 24 hours and transferred to the growth medium. When the roots reached 0.5–1 cm in a length, they were planted in several substrates, perlite, a peat-perlite mixture (1:1), triple peat-perlite-peat, wood shavings, and wood shavings -peat substrates.

Cultivation information. After sterilization, explants were grown for *in vitro* cultivation at 24-26°C, 70-80% relative humidity, a 16/8 h photoperiod, and varying light intensities of 1300-8000 lux.

Experimental schemes for optimizing nutrient medium. The experiment was conducted in three directions to study the effect of nutrient medium on microclonal propagation. In the first direction, the effect of KH_2PO_4 concentration on *in vitro* root formation was studied.Three variants wee compared: standart MS medium, as the control, ½ KH_2PO_4 and ⅔ KH_2PO_4. Each variant was carried out in 3 biological replicates with 25 explants. In the second direction, nine different nutrient medium variants were tested, which provided for modifying the concentrations of KNO_3 and NH_4NO_3 separately and in combination. In the third direction, the effect of different concentrations of sucrose as a carbon source (0; 7.5; 10; 15; 20; 30; 40 g/L) on microclonal development parameters was studied, with 60 explants were used for each variant.

Optimization of the light regime. In the study, four intensity levels of LED light sources were used: 18.5, 37.0, 46.25, and 92.5 μmol m^{-2} s^{-1}. Three spectral variants were tested: red light at 622–780 nm, blue light at 455–492 nm, and combined red–blue light at 660 + 450 nm in a 3:1 ratio. Cultivation was carried out for 4 weeks. Shoot length, number of leaves, biomass accumulation, and overall morphological condition were evaluated on a weekly basis.

Rooting stage. For rooting, two variants of a three-component gradient nutrient medium rooting complex were prepared: variant A, consisting of 1 mg/L IAA / 2 mg/L IAA in ⅔N MS medium, and variant B, consisting of 0.5 mg/L IAA / 2 mg/L IAA + 1 mg/L IBA in ⅔N MS medium. Four experimental groups, A, A1, B, and B1, were established using whole plantlets and single-node explants. After 2 weeks, rooting frequency, number of roots, and root length were evaluated. Successfully rooted plantlets were transferred to a peat:perlite:soil substrate at a ratio of 1:1:1, adapted to artificial climate conditions under transparent covers, and subsequently planted in the experimental area of the Institute of Molecular

Biology.

Phylogenetic analyses. High-quality genomic DNA was isolated from 21 grapevine samples using an "*SDS-based protocol, with A_{260}/A_{280} values ranging from 1.7 to 2.0 and DNA concentration of ≥500 ng/μL*"[4]. RAPD, ISSR, and SSR marker systems were used to assess genetic polymorphism. The UPGMA dendrogram was constructed using "*MVSP 3.1 software*"[5]. Principal Component Analysis (PCA) was also performed in the same program, while the *ape*, *phangorn*,[6] and *factoextra* [7]packages in the R environment were used for SSR-based phylogenetic analysis. "*RP* "[8]and "*PIC*"[9] values were calculated for each primer using the relevant standard formulas.

Statistical analysis. Statistical processing of the experimental results was performed using "*GraphPad Prism*"[10] software. One-way and two-way analysis of variance, "*ANOVA*"[11], were applied to compare the variants, and the significance threshold was set at $p < 0.05$. The results were expressed as mean ± standard deviation, M±SD, and graphical materials were presented as histograms. All analyses were carried out with at least 3 biological × 3 technical replicates.

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[10] GraphPad Software. GraphPad Prism version 9.5.1 for Windows. – San Diego, California, USA, 2023

[11] Zar J.H. Biostatistical Analysis. – 5th ed. – Upper Saddle River, New Jersey: Pearson Prentice Hall, 2010. – 944 p

CHAPTER III
RESEARCH RESULTS AND DISCUSSION

3. Optimization of *in vitro* propagation, morphogenesis, and developmental parameters of the Shirvanshahi grapevine variety

3.1. Preparation of donor plant material for micropropagation

Sterilization and rooting stages enabled the establishment of healthy plantlets. After mechanical wounding was applied to the lower part of the last node, the samples were incubated in an IBA solution for 24 hours, subsequently kept in distilled water, and then placed into growth vessels. Leaf buds were observed during the first week, leaves during the second week, and initial roots by the end of the third week. In the fourth week, when root length reached 1.0–1.5 cm, the plants were transferred to substrates. A comparative evaluation of six substrate variants showed that the peat:perlite (1:1) and peat–perlite–peat (2:1) substrates were characterized by the highest rates of adaptation and vegetative development. Thus, the combination of peat and perlite was determined to be the optimal substrate for *in vitro* adaptation of the Shirvanshahi grapevine variety (Figure 1).

Figure 1. Acclimatization stage of *in vivo* grapevine plants transferred to different substrates

3.2. Regulation of nitrogen compounds during *in vitro* cultivation of the Shirvanshahi grapevine variety

The effects of reducing KNO_3 and NH_4NO_3 concentrations in the MS nutrient medium, both separately and in combination, on the *in vitro*

development of the Shirvanshahi variety were investigated. A decrease in KNO_3 concentration led to a reduction in all morphometric parameters; in the ⅕ variant, shoot length decreased to 2.45 cm and leaf area to 1.20 cm^2, confirming the important role of nitrate nitrogen in vegetative development. At ¾ NH_4NO_3 concentration, shoot length (3.90 cm) exceeded that of the control variant (3.85 cm). However, at lower concentrations, disruption of the ionic balance of the medium significantly slowed morphogenesis, and shoot length decreased to 1.20 cm in the ⅕ variant (Figure 2). The highest shoot length (5.20 cm), number of leaves (7.20), and biomass (560.0 mg) were recorded in the combined variant in which both nitrogen forms were simultaneously reduced to ¾ of their standard concentration. The results of one-way ANOVA ($P < 0.0001$) confirmed the high statistical significance of the observed differences. Thus, the ¾ NH_4NO_3 + ¾ KNO_3 combination was identified as the optimal nitrogen regime for micropropagation of the Shirvanshahi variety.

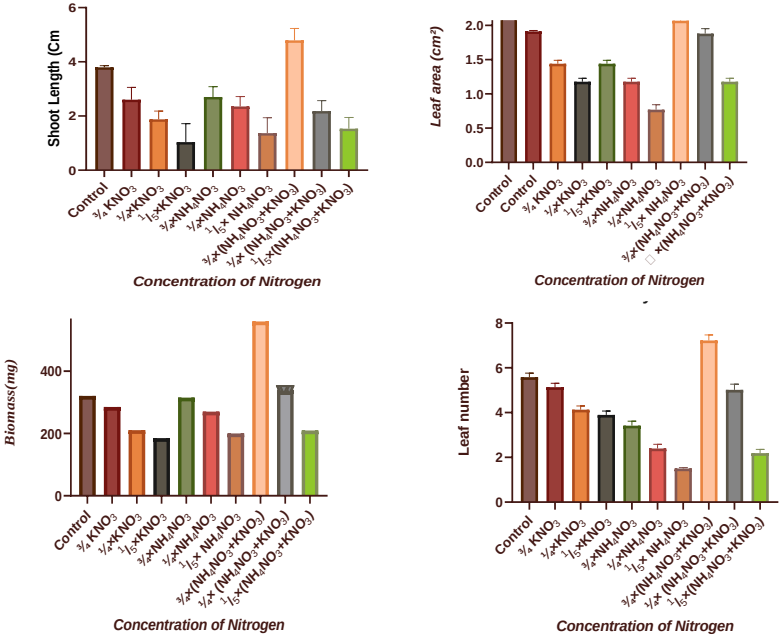

Figure 2. Effect of different concentrations of NH_4NO_3 and KNO_3 on shoot length, leaf area, biomass, and leaf number of the Shirvanshahi grapevine variety under *in vitro* conditions

3.3 Effect of phosphorus level modification in MS nutrient medium on the morphogenesis of grapevine microclones

The effects of three KH_2PO_4 concentration variants in MS medium -MS I, standard; MS II, ½ KH_2PO_4; and MS III, ⅔ KH_2PO_4 - on root development of Shirvanshahi grapevine microclones were investigated. In the MS II variant, the highest number of lateral roots (6.40 ± 0.20), root weight (0.48 g), and root/shoot ratio (73.10%) were recorded. Under reduced phosphorus conditions, the allocation of resources toward root development was evaluated as an adaptive morphogenetic strategy. In MS I, shoot length was predominant, reaching 6.80 cm (Figure 3). The results of two-way analysis of variance confirmed the high significance of all factors ($P < 0.0001$). Thus, optimization of phosphorus levels was identified as an important tool for improving the micropropagation protocol.

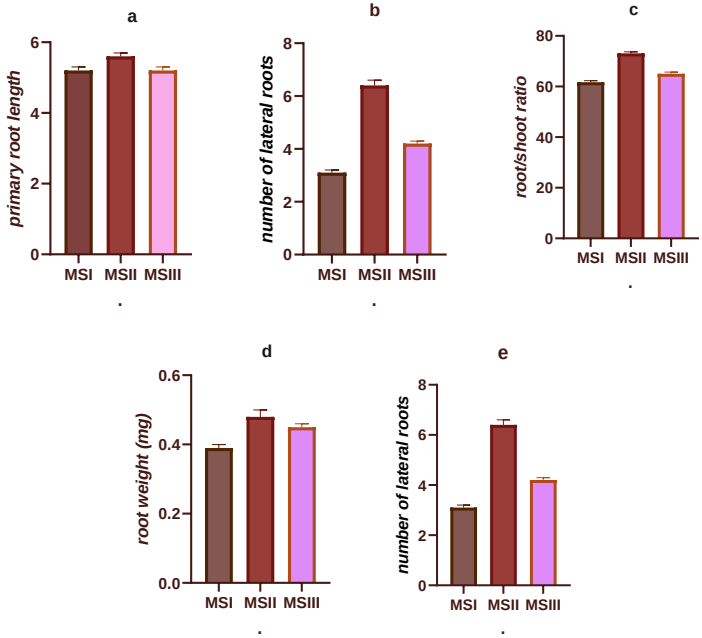

Figure 3. Effect of reduced KH_2PO_4 concentration in MS nutrient medium on *in vitro* rooting parameters: a – primary root length; b – number of lateral roots; c – root/shoot ratio;

d – root weight; e – number of lateral roots.

3.4. Development of Shirvanshahi grapevine microclones depending on sucrose concentration in the nutrient medium

Investigation of the effect of different sucrose concentrations (0–40 g/L) on the *in vitro* micropropagation of the Shirvanshahi grapevine variety showed that morphogenetic processes were almost not observed in the sucrose-free medium, whereas within the concentration range of 30–40 g/L, shoot formation reached 100%, and the mean number of shoots increased to 8.8 - 9.2 (Figure 4). These results indicate that sucrose acts not only as an energy substrate, but also as an important metabolic component involved in the regulation of cell division and differentiation processes. The results of one-way analysis of variance ($P < 0.0001$; $R^2 \approx 0.79$) confirmed the high statistical reliability of the observed differences and the strong effect of the sucrose factor on developmental parameters. Thus, 30–40 g/L sucrose was identified as the optimal carbon regime for the micropropagation of the Shirvanshahi variety.

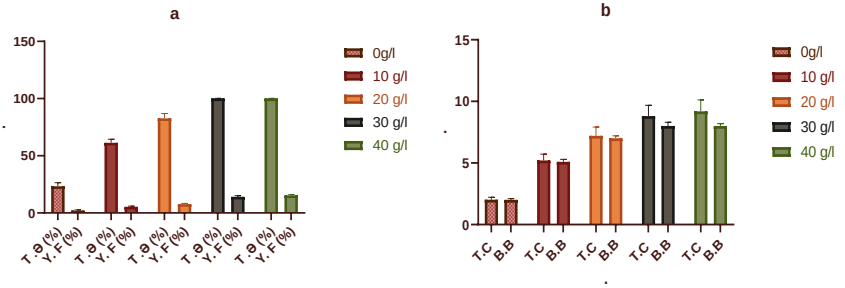

Figure 4. Effect of sucrose concentration on the *in vitro* development dynamics of explants: a – shoot formation and contamination parameters; b – number of shoots and growth score.

3.5. Effect of light intensity and spectral composition on *in vitro* morphogenesis of the Shirvanshahi grapevine variety

The effects of different LED light intensities (18.5, 37.0, 46.25, and 92.5 µmol m^{-2} s^{-1}) and spectral regimes (red, blue, B+R combination, and white light) on the *in vitro* morphogenesis of the Shirvanshahi grapevine variety were comprehensively investigated. At an intensity of 18.5 µmol m^{-2} s^{-1}, the plants exhibited weak development due to insufficient energy

supply, whereas at 92.5 µmol m^{-2} s^{-1} all plants died by the third week as a result of photooxidative stress. Optimal development was recorded at an intensity of 46.25 µmol m^{-2} s^{-1}, at which stem elongation, leaf number, and biomass accumulation reached their maximum values (Figure 5). The results of two-way analysis of variance ($P < 0.0001$) showed that the light intensity factor alone accounted for 51.75% of the total variation.

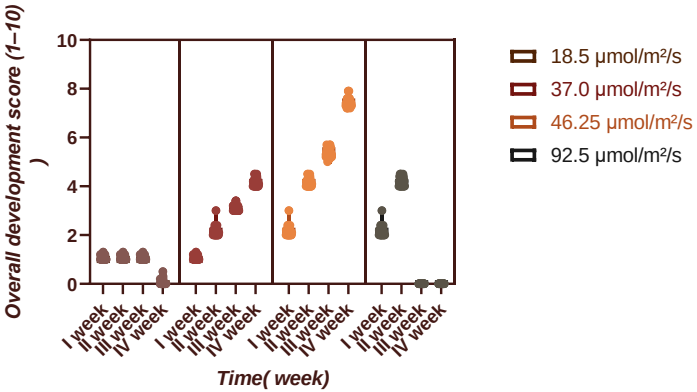

Figure 5. *In vitro* development dynamics of the Shirvanshahi grapevine variety under different light intensities.

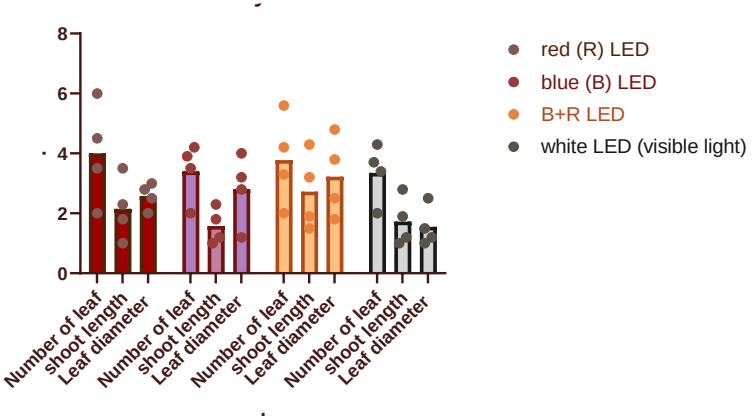

Figure 6. Effect of different LED light spectra on the development of *in vitro* morphological parameters of the Shirvanshahi grapevine variety.

Studies on the effect of spectral composition showed that the highest morphological parameters - leaf number (5.6), stem length (4.3 cm), and leaf diameter (4.8 mm) - were recorded at the end of the fourth week under a LED combination of 70% red and 30% blue light (Figure 6). The results of two-way analysis of variance confirmed the statistical significance of the spectral factor (P = 0.0008), which accounted for 28.06% of the total variation. Thus, a spectral ratio of 70% R + 30% B at an intensity of approximately 46.25 μmol m^{-2} s^{-1} was identified as the optimal light regime for *in vitro* micropropagation of the Shirvanshahi variety.

3.6. Rooting and *in vitro* adaptation

The rooting stage is one of the most important phases of micropropagation and directly determines the *in vitro* adaptation potential of explants. In woody plants, particularly in species belonging to the genus *Vitis*, *in vitro* rooting is characterized by several limitations, including strong genotype dependence, low morphogenetic capacity of tissues, and hormonal imbalance.To address these challenges, a three-component gradient nutrient medium complex was developed for the first time within the framework of this study, based on the spatial and temporal differential effects of hormonal and mineral components. A hormone-free mineral base was applied in the lower layer of the system, a low dose of IAA in the middle layer, and a high auxin background consisting of 2/3 N + ½ P MS nutrient medium supplemented with a combination of IAA and IBA in the upper layer. In the study, two modifications of the system, Variant A and Variant B, were comparatively evaluated using whole microclones and single-node explants. The maximum rooting parameters were obtained when whole microclones were subcultured onto Variant B medium: rooting frequency was 74.0 ± 5.0%, the number of lateral roots was 8.6 ± 0.3 per explant, and root length reached 7.8 ± 0.2 cm. In Variant A, these parameters were 55.33 ± 3.51%, 3.8 ± 0.3 per explant, and 4.6 ± 0.4 cm, respectively (Figure 7). In single-node explants, rooting frequency was considerably lower in both variants, 27.0 ± 4.0% in Variant A and 32.7 ± 2.1% in Variant B, confirming that explant type has a decisive effect on rooting efficiency (Figure 8).

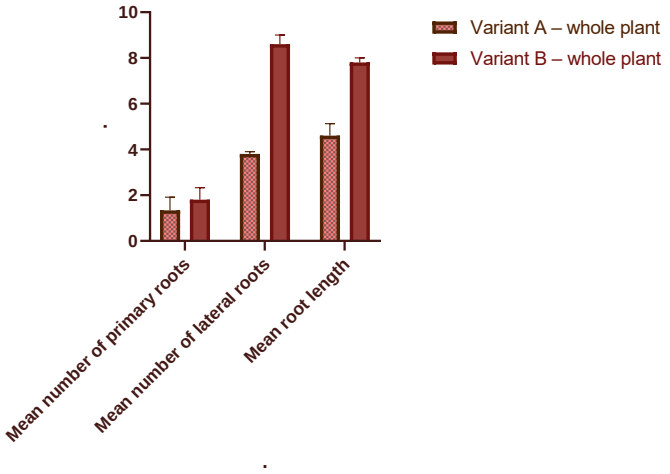

Figure 7. Effect of A and B nutrient media on the morphological parameters of the root system in whole-plant explants

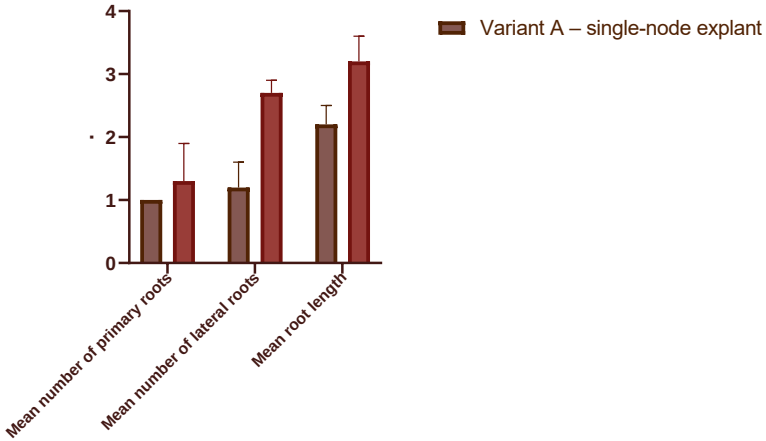

Figure 8. Effect of A and B nutrient media on the morphological parameters of the root system in single-node explants

The superiority of Variant B is substantiated by the synergistic effect of IAA and IBA: "*IBA is converted into biologically active IAA through*

the β-oxidation pathway, thereby ensuring localized enhancement of auxin signaling in the rooting zone."[12][13] Meanwhile, exogenous IAA induces a rapid hormonal response and stimulates the induction process.

Two-way analysis of variance (two-way ANOVA) confirmed that both the nutrient medium variant and morphogenetic parameters had a statistically significant effect on root system development in whole microclones ($p < 0.0001$) as well as in single-node explants ($p = 0.0002$).

Thus, by establishing a controlled, staged model of root formation, the three-component gradient complex proved itself to be an effective rooting protocol that differs fundamentally from conventional homogeneous auxin-containing media.

3.7. Plant adaptation and transfer to *in vivo* conditions

"The acclimatization of microplants rooted under in vitro conditions to the in vivo environment represents the final and, at the same time, a critical stage of micropropagation, involving the transition of plants from an artificial, aseptic environment with stable nutrient supply to variable external conditions."[14]

In our study, after completion of the rooting stage, the microplants were cleaned of agar adhering to the root system and transferred to a previously autoclave-sterilized perlite–peat (1:1:1) substrate characterized by high acclimatization capacity. During the initial stage of *ex vitro* acclimatization, the plants were maintained under glass covers for 9 days in order to create mini-greenhouse conditions. In the next step, the covers were gradually removed, allowing the plants to adapt progressively to open atmospheric conditions.Due to the unstable functioning of stomata in microplants formed under *in vitro* conditions, namely the incomplete establishment of transpiration control, the maintenance of high

[12] Damodaran S., Strader L.C. Indole 3-Butyric Acid Metabolism and Transport in *Arabidopsis thaliana* // *Frontiers in Plant Science*. – 2019. – Vol. 10. – Art. 851
[13] Frick E.M., Strader L.C. Roles for IBA-derived auxin in plant development // *Journal of Experimental Botany*. – 2018. – Vol. 69, No. 2. – P. 169–177.
[14] Pospíšilová J., Tichá I., Kadleček P., Haisel D., Plzáková Š. Acclimatization of micropropagated plants to *ex vitro* conditions // Biologia Plantarum. – 1999. – Vol. 42, No. 4. – P. 481–497.

humidity and a balanced substrate during the initial acclimatization period was of decisive importance. During the acclimatization process, partial necrosis of the aerial parts formed at the *in vitro* stage was observed. At the subsequent stage, the development of new shoots from the basal meristematic zone was recorded (Figure 9).

After the plants reached a length of 50–60 cm, they were successfully transferred to open soil conditions. Thus, the applied stepwise gradient scheme, including transfer to a sterile substrate, maintenance under a closed microclimate, gradual transition to open conditions, and subsequent transfer to soil, was identified as a scientifically grounded and practically efficient protocol for the *ex vitro* acclimatization of microplants of the Shirvanshahi variety.

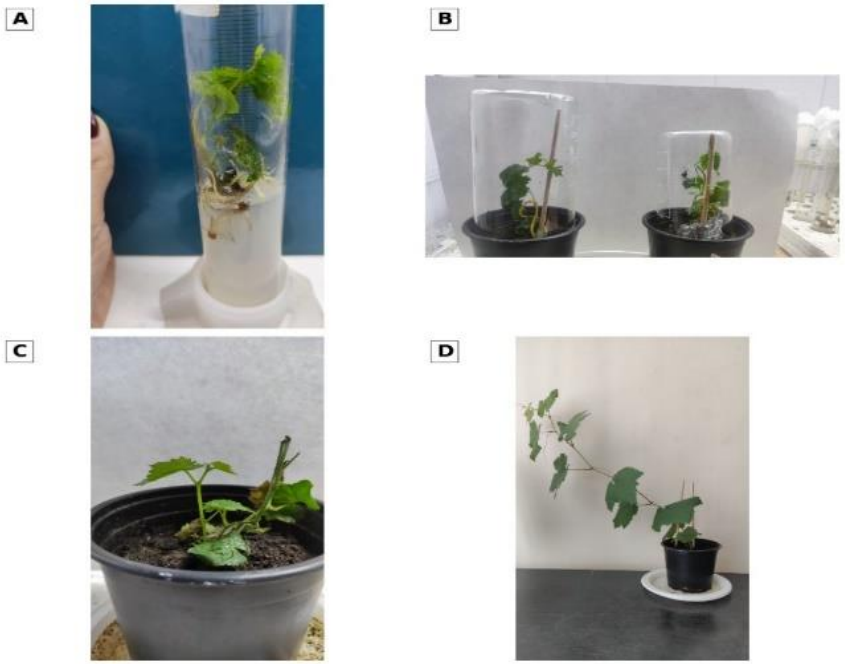

Figure 9. Sequential acclimatization stages observed in microplants of the Shirvanshahi variety

Figure 10. The Shirvanshahi grapevine variety transferred to the natural growing environment and continuing vegetative development

CHAPTER IV
Analysis of genetic similarity among the Shirvanshahi variety, local and wild grapevine samples based on RAPD, ISSR, and SSR molecular markers

4.1. Analysis of genetic similarity among the Shirvanshahi variety, local and wild grapevine samples based on RAPD markers

The genetic diversity of 21 grapevine samples investigated using 16 RAPD primers was evaluated. A total of 182 bands were detected, of which 85.7% were polymorphic. The mean values of RP and PIC calculated for RAPD markers, 3.34 and 0.43, respectively, indicated that the primers had moderate to high informativeness in detecting intergenotypic polymorphism. In the UPGMA dendrogram constructed based on RAPD data, the samples were grouped into two main clusters. Although a tendency toward genetic differentiation between wild and cultivated forms was observed, the high similarity shown by some samples confirmed that these groups were not completely isolated from each other.

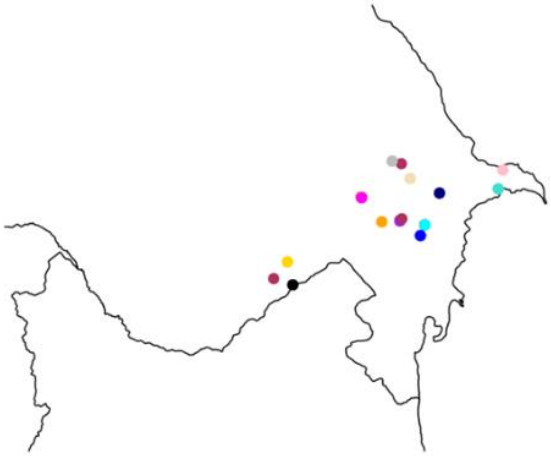

Figure 11. Schematic representation of the geographical origin of the Shirvanshahi variety, local grapevine samples, and wild grapevine samples.

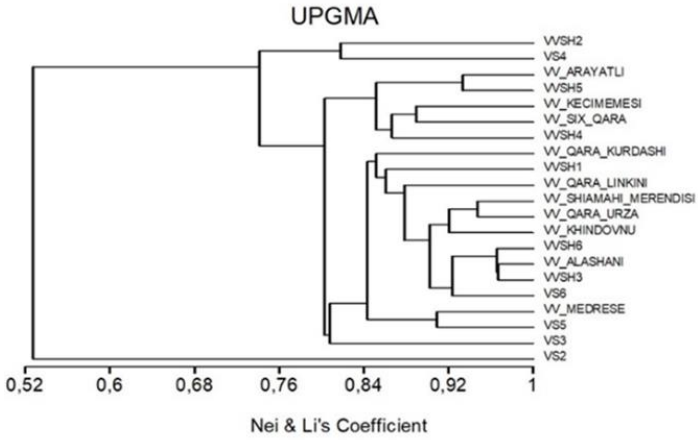

Figure 12. RAPD dendrogram of grapevine samples constructed using MVSP software

The highest genetic similarity was recorded between VS6 and VVSH3, 0.954, while the lowest similarity was observed between VS2 and VV_Qara_Urza, 0.458. The overall mean similarity was

71.25%. Samples numbered 1–5, representing *Vitis vinifera* L. subsp. *Sylvestris*, formed a separate cluster, with similarity values ranging from 0.512 to 0.852.

4.2. Genotyping of grapevine samples using ISSR markers and analysis of genetic similarity

The results obtained with the 12 ISSR primers were consistent with those of the RAPD analysis; however, a higher level of polymorphism was detected, with a polymorphism information content (PIC) of ≥ 0.30. ISSR markers more accurately reflected the genetic differentiation among cultivated genotypes. The within-cultivar polymorphism of the Shirvanshahi variety reached 52.5%, indicating a high level of genetic diversity within the cultivar. The ISSR dendrogram constructed using the UPGMA method and the Nei–Li similarity coefficient showed that the samples were grouped into two major clusters. Although wild and cultivated forms exhibited a certain degree of genetic differentiation, they did not form completely distinct groups because of the high genetic similarity observed among some of the samples (Figure 13).

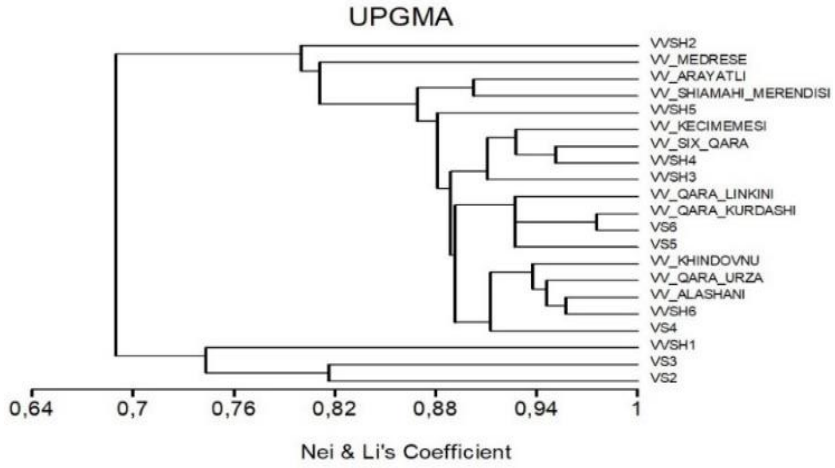

Figure 13. UPGMA dendrogram of grapevine samples obtained using MVSP software.

The highest genetic similarity was recorded between VV_Qara_Kurdashi and VS6 (97.6%), while the lowest similarity was observed between VVSH2 and VS2 (52.5%). The overall mean similarity was 75.05%. The close relationship between the VV_Qara_Kurdashi–VS6 pair may indicate shared geographical and ecological conditions, as well as the presence of historical-genetic links between wild and cultivated forms.PCA analysis based on ISSR markers generally confirmed the dendrogram results. Although most of the samples were located within a common genetic proximity area, the separation of some samples from the main group indicated the presence of marker-specific genetic differences. The consistency between the dendrogram and PCA results confirms that ISSR markers are a reliable tool for determining genetic similarity and differentiation among grapevine samples

4.3. Genotypic and phylogenetic analysis of grapevine samples based on microsatellite (SSR) markers

SSR markers demonstrated higher informativeness than RAPD and ISSR in detecting allelic variations and somatic mutations. PIC values ranged from 0.45 to 0.78. The presence of (GA)n microsatellite motifs for the VVIp31 marker and (CA)n microsatellite motifs for the VVIn73 marker was confirmed in all 21 grapevine samples. The number of repeats varied from 12 to 20 for VVIp31 and from 6 to 10 for VVIn73. According to the genetic distance matrices, the lowest and highest values for the VVIp31 marker were recorded between VVSH6–VS3 (0.9%) and VVSH5–VS4 (30.4%), respectively, while for VVIn73, the corresponding values were observed between VS6–VV_Qara_Linkini (0.6%) and VV_Madrasa–VV_Qara_Urza (10.97%) (Figure 14). These results indicate that the VVIp31 locus exhibited a broader range of variation and higher discriminatory capacity among the studied samples. In contrast, genetic distance values for the VVIn73 marker were observed to vary within a relatively narrower range.

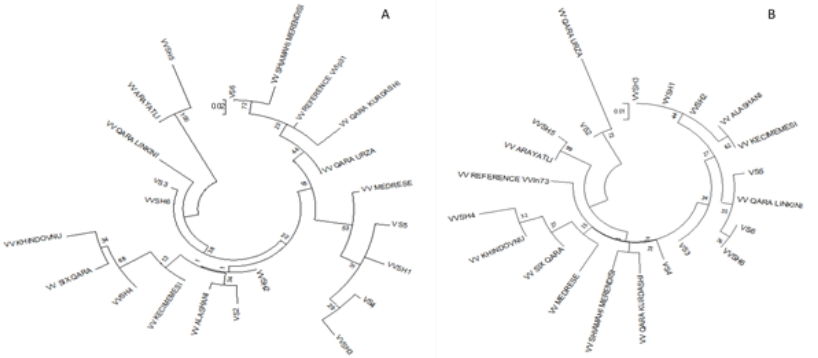

Figure 14. Dendrograms of grapevine samples based on VVIp31 (A) and VVIn73 (B) markers, constructed using MEGA 6.0 software.

Dendrograms constructed based on the ML (Maximum Likelihood) topology showed that *Vitis vinifera* L. subsp. *sylvestris* and *Vitis vinifera* L. subsp. *vinifera* samples did not form completely separated groups for either SSR marker, and in some cases exhibited shared or closely related branching patterns (Figure 15). This result suggests the possible existence of genetic relationships between wild and cultivated grapevine forms.

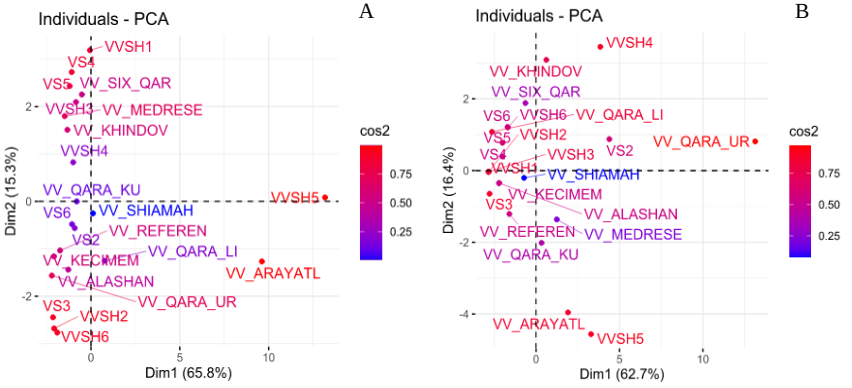

Figure 15. ML dendrograms of samples based on VVIp31 (A) and VVIn73 (B) markers, constructed in the R programming environment using the *ape* and *phangorn* packages.

Principal Coordinate Analysis (PCoA) was performed based on the genetic distance matrices obtained for the SSR markers. For the VVIp31 marker, the proportions of variation explained by the first two coordinate axes were 65.8% and 15.3%, respectively, while for the VVIn73 marker, the corresponding values were 62.7% and 16.4%. These results indicate that, for both markers, the first two coordinates explained the major part of the total genetic variation. The PCoA results generally supported the dendrogram data. Although a certain degree of differentiation was observed between *Vitis vinifera* L. subsp. *sylvestris* and *Vitis vinifera* L. subsp. *vinifera* samples in the plots, complete separation between the groups was not recorded, indicating that wild and cultivated grapevine forms are genetically related.

In particular, the clear separation of the VVSH5 sample from the other samples in both PCoA plots was noteworthy; this may be explained by its marker-specific allelic variation. Thus, the use of two loci in SSR analysis enabled the assessment of genotypic differentiation and marker-specific relationships of genetic proximity between wild and cultivated grapevine forms.

4.4. Comparative analysis of molecular-genetic diversity and phylogenetic relationships of grapevine samples based on RAPD, ISSR, and SSR markers

Molecular-genetic analysis of grapevine genotypes based on RAPD, ISSR, and SSR markers showed that the overall genetic similarity among the studied samples was 71.25% for RAPD and 75.05% for ISSR. The dendrograms and PCA results constructed using both dominant marker systems revealed a certain tendency of grouping between wild and cultivated grapevine forms. However, the high level of similarity observed between some samples, including 97.6% similarity between VV_Qara_Kurdashi and VS6, confirmed that these two groups were not separated as completely isolated genetic units.

The ML topology and PCoA analysis performed based on SSR markers, VVIp31 and VVIn73, produced similar results, and the VVIp31 locus was found to exhibit a broader range of variation and higher discriminatory capacity. The high genetic proximity obtained among the VVSH1–VVSH6 samples belonging to the Shirvanshahi variety

indicated that this variety has preserved its main genetic structure despite being cultivated in different geographical areas.

The differentiation of the VVSH5 and VS3 samples from the other genotypes for both SSR markers revealed the presence of marker-specific local genetic variation in these samples. While the broad genetic variation observed in wild grapevine populations can be explained by evolutionary processes occurring in natural populations and ecogeographical isolation, cultivated varieties were characterized by a more homogeneous genetic structure formed as a result of long-term selection and vegetative propagation.

Consequently, the combined application of RAPD, ISSR, and SSR marker systems complemented one another and provided a scientific and practical basis for the molecular characterization of the Shirvanshahi variety and other local grapevine genetic resources, as well as for the conservation of genetic resources and their use in future breeding programs.

FINAL ANALYSIS OF THE RESULTS OBTAINED IN THE RESEARCH

The dissertation is devoted to the study of the biotechnological propagation, assessment of genetic diversity, and molecular-genetic characterization of the Shirvanshahi indigenous grapevine variety within a unified scientific framework. The research was based on the necessity of conserving and mass restoring the Shirvanshahi variety, which has almost disappeared from production vineyards but has retained high breeding and genetic potential.The aim of the study was not limited solely to the development of a micropropagation protocol, but also included the determination of the phylogenetic relationships of the variety with local and wild grapevine genotypes. The sucrose concentration of 30–40 g/L, the balanced ratio of NH_4NO_3 and KNO_3, as well as the regulation of KH_2PO_4 levels, promoted more stable development and high regeneration potential in Shirvanshahi microclones. These findings justify the assessment of MS nutrient medium not as a static system for the Shirvanshahi variety, but as a cultivation base that can be functionally modified. Experiments on

light regimes showed that 70% red and 30% blue LED light at medium intensity (~46 μmol m^{-2} s^{-1}) ensured morphogenetic balance, compactness of the leaf apparatus, and stability of shoot development. At the rooting stage, the three-component gradient complex based on the combination of IAA and IBA under a reduced nitrogen background produced more effective results in terms of successful root system formation. The *ex-vitro* acclimatization stage increased the stress tolerance of microplants and the stability of their subsequent vegetative development. The combined application of RAPD, ISSR, and SSR marker systems proved to be an effective and scientifically validated approach for the molecular characterization of the Shirvanshahi variety. Thus, the conducted research established a scientific and practical basis for the conservation of the Shirvanshahi variety as a genetically valuable indigenous grapevine genotype, its propagation as healthy planting material, and its future use in breeding, gene pool conservation, and viticulture programs.

CONCLUSIONS

1. For the first time, it was established that the *in vitro* development of *Vitis vinifera* L. Shirvanshahi microclones is determined not only by the total amount of nitrogen, but also by the ratio of nitrate and ammonium forms. It was shown that optimal morphological development occurs under conditions of a balanced simultaneous reduction of both nitrogen forms (NH_4NO_3 + KNO_3), which ensures cell division and the formation of the photosynthetic surface. Under these conditions, the maximum morphometric parameters were obtained: shoot length — 5.20 cm; number of leaves — 7.2; $F(9,240) = 170.1$; $P < 0.0001$; $R^2 = 0.8645–0.9819$. The proposed nitrogen regime can be considered a scientifically and practically grounded model for the effective regulation of morphogenesis during micropropagation. [1,6,8,9,13]
2. For the first time, it was revealed that phosphorus level plays a key role in the formation of root system architecture in *Vitis vinifera* L. Shirvanshahi microclones obtained under *in vitro* conditions. Phosphorus deficiency weakened primary root elongation and

stimulated lateral root development, resulting in adaptive reorganization of the root system and increased efficiency of phosphorus assimilation. Statistical analyses showed that this effect was highly significant ($P < 0.0001$), with 61.54% of the total variation attributable to the main factor and 32.76% to the interaction effect. [5,7,8,9]

3. LED light intensity was confirmed to be a fundamental and limiting factor in the *in vitro* morphogenesis of the Shirvanshahi grapevine variety. The maximum developmental parameters were obtained at an intensity of 46 μmol m^{-2} s^{-1}. At 18.5 μmol m^{-2} s^{-1}, morphogenetic activity and developmental parameters declined, whereas an illumination intensity of 92.5 μmol m^{-2} s^{-1} caused explant destruction and death. It was found that integral developmental parameters were mainly determined by light intensity, while the time factor and interactions between factors were statistically insignificant. [2,17,18]
4. For the first time, using a photobiotechnological approach, the effect of the spectral composition of light as an ecophysiological factor on the vegetative development and morphogenesis of the Shirvanshahi grapevine variety was revealed. The optimal parameters of morphogenetic processes were obtained under medium light intensity (~46 μmol m^{-2} s^{-1}) using 70% red + 30% blue LED light, whereas the effects of cultivation duration and its interaction with the spectrum were not statistically significant. [2,12,17]
5. The three-component gradient nutrient medium complex, applied for the first time in the Shirvanshahi grapevine variety, was presented as a new scientifically grounded approach that ensures the stepwise regulation of rooting through the spatial and temporal differential distribution of nutritional and hormonal components. It was determined that, in whole microclone explants, Variant B nutrient medium, enriched with a combination of IAA and IBA under a reduced nitrogen background, provided the highest results in terms of rooting frequency (74.0 ± 5.0%), lateral root formation (8.6 ± 0.3 roots per explant), and root length (7.8 ± 0.2 cm), and

was characterized by statistically significant differences compared with the other experimental variants ($p < 0.05$). [7,14,15,19]

6. It was determined that the three-component gradient complex forms a controlled model of rooting based not on the effect of individual components, but on their synergistic and stepwise action, and therefore differs fundamentally from conventional *in vitro* rooting approaches. Two-way ANOVA confirmed that the interaction among the nutrient medium factor, explant type, and morphogenetic parameters was statistically significant ($p < 0.05$–0.0001), indicating that the effectiveness of the proposed system is based on multifactorial mechanisms. [3,14,15]
7. The sequential application of RAPD, ISSR, and SSR marker systems quantitatively characterized the genetic diversity of 21 grapevine samples distributed in Azerbaijan, including 16 samples of *V. vinifera* L. subsp. *vinifera* and 5 samples of *V. vinifera* L. subsp. *sylvestris*. The mean genetic similarity was 71.25% for RAPD markers and 75.05% for ISSR markers. Cluster, PCA, and PCoA analyses revealed a certain tendency toward genotypic differentiation between the two taxa. However, the high similarity observed between individual wild–cultivated pairs, including VS6–VVSH3 (0.954) and VV_Qara_Kurdashi–VS6 (97.6%), showed that these two taxa do not constitute genetically fully isolated and closed units. This finding indicates that, in accordance with evolutionary biological regularities of domestication processes, regional wild populations participated as ancestral gene pool sources in the formation of local cultivated grapevine varieties. [4,16]
8. For the first time, the indigenous Shirvanshahi grapevine variety was comparatively studied at the molecular level together with local and wild grapevine samples distributed in areas close to its cultivation range. It was revealed that the variety has strong genetic affinity with wild forms (*V. vinifera* subsp. *sylvestris*), with a mean similarity of 91.1%. Thus, the possible origin of the Shirvanshahi variety from local wild grapevine forms was confirmed at the molecular level. The results of the dissertation characterize this

variety as a valuable genetic resource for future breeding programs. [4,10,16]

PRACTICAL RECOMMENDATIONS

1. It is recommended that the developed micropropagation protocol be applied in scientific research institutions and specialized nurseries of the Republic of Azerbaijan as an effective biotechnological tool for the conservation, rapid propagation, and efficient use of indigenous grapevine varieties.
2. It is recommended that the identification system developed on the basis of RAPD, ISSR, and SSR markers be applied in scientific research institutions and genetic resource collections for the molecular-genetic passporting of indigenous and wild grapevine genotypes**.**

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6. Sadigova A.A., Mammadova M.H., Garagozov T.H., Huseynova I.M. Strategic Adjustment of Nitrogen Compounds in the *In vitro* Cultivation of Shirvan Shahi (*Vitis vinifera* L.) // Advances in Biology & Earth Sciences, – December, 2025, v. 10, No 3, – p. 397-408. – https://doi.org/10.62476/abes.103397
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The defense of the dissertation will be held on "11" September 2026 at 11-00 at the meeting of the FD 1.07 Dissertation Council operating under the Institute of Molecular Biology of the Ministry of Science and Education of the Republic of Azerbaijan, under the Higher Attestation Commission under the President of the Republic of Azerbaijan.

Address: 11 Izzat Nabiyev Street, Baku, AZ1073, Republic of Azerbaijan.

The dissertation is available for review at the library of the Institute of Molecular Biology of the Ministry of Science and Education of the Republic of Azerbaijan.

The electronic version of the abstract has been posted on the official website of the Institute of Molecular Biology of the Ministry of Science and Education of the Republic of Azerbaijan: https://imbb.az/az.

The abstract was sent to the required addresses on "8" august 2026.

Signed for publication: 07.08.2026

Paper format: A5

Volume: 41770

Copies: 20