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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

STUDY OF EPIGENETIC CHANGES IN WHEAT CULTURE ISOLATED UNDER *IN VITRO* CONDITIONS

Speciality:	2422.01 – Biotechnology (including bionanotechnologies)
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Applicant:	**Gunay Ilman Ismayilova**

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The dissertation work was performed at the Plant Biotechnology Laboratory of the Institute of Molecular Biology and Biotechnologies of the Ministry of Science and Education of the Republic of Azerbaijan and the Plant Tissue Culture Laboratory of Yıldız Technical University of the Republic of Turkiye.

Scientific supervisors: PhD in Biology
Tofig Husni Garagozov

PhD in Biology, Associate Professor
Mahira Heybat Mammadova

Official opponents: Full Member of ANAS
Mammad Ahad Salmanov

Doctor of Biological Sciences,
Associate Professor
Saib Gurban Gulahmadov

PhD in Biology
Ulker Vagif Neymatova

BFD 1.07/1 Dissertation Council operating under the Institute of Microbiology of the Higher Attestation Commission under the President of the Republic of Azerbaijan.

Chairman of the
Dissertation Council: Doctor of Biological Sciences, Professor,
Correspondent Member of ANAS
Panah Zulfugar Muradov

Scientific secretary of the
Dissertation Council: PhD in Biology
Vafa Yashar Hasanova

Chairman of the Scientific
Seminar: Doctor of Biological Sciences, Associate Professor
Vafa Khalil Gasimova

INTRODUCTION

Relevance of the topic and degree of development. It is well known that "*plant resistance to abiotic stresses is considered a genetically complex reaction and is polygenic in nature"*[1] Also, *"the effect of stressors at different stages of development directly affects the course of molecular, biochemical, and physiological processes in the plant, which is manifested in a decrease in product quality and overall productivity*"[2]. Today, tissue culture methods are used to obtain agricultural plants resistant to various abiotic stress factors. In many countries of the world, "*resistant varieties of agricultural plants adapted to specific soil and climatic conditions against various abiotic stresses have been obtained*"[3] using this method. In Azerbaijan, work is also being carried out in this direction with tissue culture in various plants, but epigenetic changes occurring during *in vitro* selection have not been studied either at the cellular level or in regenerative plants.

It should be borne in mind that explants isolated from multicellular organisms contain various differentiated cells. They perform different functions by responding differently to environmental signals. It is these epigenetic factors that provide "cellular memory" in living organisms, including tissues obtained from these organisms. Unlike mutations, "*epigenetic changes are reversible and can be controlled"*[4]. This facilitates selection work aimed at obtaining

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[1] Zhang, Y.; Xu, J.; Li, R.; Ge, Y.; Li, Y.; Li, R. Plants' Response to Abiotic Stress: Mechanisms and Strategies. *Int. J. Mol. Sci.* 2023, *24*, 10915. https://doi.org/10.3390/ ijms241310915

[2] Imran, Q.M.; Falak, N.;Hussain, A.; Mun, B.-G.; Yun, B.-W. Abiotic Stress in Plants; Stress Perception to Molecular Response and Role of Biotechnological Tools in Stress Resistance. *Agronomy* 2021, *11*, 1579. https://doi.org/10.3390/ agronomy11081579

[3] Wijerathna-Yapa, A.; Hiti-Bandaralage, J. Tissue Culture—A Sustainable Approach to Explore Plant Stresses. *Life* 2023, *13*, 780. https://doi.org/10.3390/ life13030780

[4] Watanabe A., Yamada Y., Yamanaka S. Epigenetic regulation in pluripotent stem cells: a key to breaking the epigenetic barrier. (англ.) // Philosophical transactions of the Royal Society of London. Series B, Biological sciences. — 2013. — Vol. 368, no. 1609. — P. 20120292.

resistant, high-yielding varieties and creates a platform for the application of experimental processes by modeling *in vitro* conditions.

Epigenetics studies heritable changes in gene activity during cell growth and division, specifically, mechanisms that do not change the nucleotide sequence in DNA and regulate gene expression. Recently, along with DNA methylation, chromatin remodeling, RNA interference, prion-like protein behavior, and X chromosome inactivation, another mechanism that affects gene expression during ontogenesis in plants has been studied - these are mobile genetic elements known as retrotransposons. Interest in retrotransposons stems from their significant representation in the genome of living organisms. Retrotransposons also play a very important role in the organization, activity and evolution of the plant genome. "*Retrotransposons affect the structure and function of the genome by altering gene function, activating different fragments in genes, and influencing epigenetic control. They can alter the transcription or processing of nearby structural genes and are also involved in gene silencing*"[5].

Studies have shown that "*the mobility of retrotransposons changes significantly during different stages of ontogenesis, particularly under stress conditions*"[6]. This leads to an increase in polymorphism in plants. The study of the mobility of retrotransposons during induced dedifferentiation, callus formation, morphogenesis and *de novo* reconstruction of plants under *in vitro* cultivation conditions creates broad opportunities to monitor and regulate polymorphism processes in plants. In this way, it is possible to obtain genetically stable plants i.e. plants with very low polymorphism, and plants prone to variability, i.e. plants with a high polymorphism index. On the other hand, the use of retrotransposons as molecular markers in *in vitro* selection studies enable precise and targeted breeding, representing a modern approach to developing high-yielding wheat varieties resistant to various stresses according to specific breeding goals.

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[5] Bennetzen, Jeffrey L.; Wang, Hao (2014). The Contributions of Transposable Elements to the Structure, Function, and Evolution of Plant Genomes. Annual Review of Plant Biology, 65(1), p. 505–530.

[6] Сормачева И.Д., Блинов А.Г. LTR-ретротранспозоны растений // Вавиловский журнал генетики и селекции. 2011. Т. 15. № 2. С. 351–381

Object and subject of the study. Local wheat varieties obtained from the Agricultural Research Institute of the Ministry of Agriculture of the Republic of Azerbaijan were used in the study. During the research, cell cultures, regenerant and intact plants of wheat genotypes grown under normal and stress conditions in the artificial climate chamber of the Plant Biotechnology Laboratory of the Institute of Molecular Biology and Biotechnologies of the Ministry of Science and Education of the Republic of Azerbaijan were used. Epigenetic analyses were conducted in the Plant Tissue Culture Laboratory of the Genetics Department, Faculty of Science, Yıldız Technical University, Republic of Türkiye.

Aims and objectives of the study. The main aim of the research was to study epigenetic changes in cell culture, regenerants, and intact plants obtained under normal and stress conditions.

In accordance with this aim, the following tasks were set:

➢ Transferring immature wheat embryos of different sizes to *in vitro* culture and obtaining callus tissue;

➢ Studying the effect of different concentrations and combinations of phytohormones on the morphogenesis process in calli;

➢ Evaluation of *in vitro* competence of genotypes and selecting samples for epigenetic analyses;

➢ Studying the effect of different concentrations of $CdSO_4$ and NaCl salts on the morphogenesis ability of wheat cell culture and obtaining regenerant plants;

➢ Detection of various retrotransposons in cell culture, regenerant plant leaves, and intact plant samples obtained under stress conditions by the IRAP marker method and comparative analysis of their polymorphism values using the Jaccard coefficient;

➢ Construction of a phylogenetic tree by UPGMA cluster analysis and determination of the genetic similarity coefficient between samples.

Research methods. The theoretical part of the study was based on an extensive and comprehensive analysis of modern literature. Biotechnological and molecular research methods were employed in

the dissertation work. Statistical analysis of all the results obtained was carried out.

The provisions of the dissertation presented for defense:

➢In plant tissue culture, genotype, explant size, phytohormone concentration, nutrient medium composition directly affect callus induction, proliferation, morphogenesis and plant reconstruction;

➢ During the cultivation of immature wheat germs, there is a negative correlation between the increasing NaCl salt concentration in the selective medium and the callus proliferation intensity and biomass accumulation dynamics;

➢ In callus, regenerant and intact plants of the studied wheat genotypes, retrotransposons specific to rice (Houba), barley (Sukkula) and soybean (Sire1) plants are present;

➢ In *in vitro* cultured callus tissue, regenerants and intact plants, the mobility of retrotransposons changes depending on the cultivation period and statistically significant differences in polymorphism values arise;

➢ The genetic similarity coefficient is different in different wheat genotypes depending on the development period.

Scientific novelty of the research: The dissertation work is the first study in Azerbaijan dedicated to the study of the dependence of the competence of various local wheat varieties exposed to stress *in vitro* on the mobility and polymorphism level of retrotransposons in cell culture, morphogenesis and plant reconstruction.

The effect of genotype, explant size, different concentrations and combinations of phytohormones on callus induction, proliferation intensity and biomass growth in local wheat varieties cultivated *in vitro* was studied.

The competence of the wheat genotypes Barakatli-95, Gobustan, Jumhuriyet-100, Gyrmyzy bugda and Nurlu-99 *in vitro* was confirmed for callusogenesis, morphogenesis and plant reconstruction.

The effect of different concentrations of NaCl and $CdSO_4$ salts on callus proliferation intensity and morphogenesis was studied.

Regenerant plants with normal, vital functions and reproductive capacity were obtained from tissue cultures of Jumhuriyet-100,

Gyrmyzy bugda and Nurlu-99 wheat varieties in 150 mM NaCl concentration.

For the first time in Azerbaijan, Houba, specific to rice plants, Sukkula, specific to barley plants and Sire1, specific to soybean plants were detected in local wheat varieties using the IRAP marker method.

For the first time, the movement activity of retrotransposons specific to barley (Sukkula), rice (Houba) and soybean plants (Sire1) was studied in calluses of Jumhuriyet-100, Gyrmyzy bugda and Nurlu-99 genotypes exposed to NaCl and $CdSO_4$ salts in leaves of intact and regenerant plants. The polymorphism values of the samples were calculated using the Jaccard similarity coefficient based on monomorphic and polymorphic bands in the electrophoregram.

For the first time, a phylogenetic tree of callus and plant samples of Jumhuriyet-100, Gyrmyzy bugda and Nurlu-99 wheat genotypes was constructed using UPGMA cluster analysis based on Houba, Sukkula and Sire1 primers.

Theoretical and practical significance of the study. It was determined that the processes of morphogenesis in isolated plant cells are interconnected with the combination, ratio and concentration of phytohormones.

For the first time, wheat varieties were evaluated *in vitro* during selection in soil and laboratory conditions as genetically stable and polymorphic varieties at the cellular, regenerant and donor plant levels based on the mobility of the Houba, Sukkula and Sire1 retrotransposons.

It was shown that the ability of the wheat genome to undergo epigenetic reprogramming leads to adaptation to changing environmental conditions during the formation of both cells, morphogenesis and regenerant plants, as well as changes in ontogenesis programs.

In the dissertation work, it was shown for the first time that artificial nutrient medium conditions with changing phytohormonal composition and stress factors cause changes in the epigenome of wheat cell culture and that the stability and lability properties of wheat varieties, i.e., the intraspecific polyvariance of plants, depend not only on genetic polymorphism but also on epigenetic events, including the

mobility of retrotransposons changes occurring during ontogenesis.

The formation of physiologically normal, reproductively capable regenerative plants at a concentration of 150 mM NaCl salt was manifested as one of the adaptive ways of epigenetic regulation in the studied wheat varieties.

The serious negative effect of cadmium, a heavy metal, on basic physiological processes such as callus formation, proliferation, morphogenesis, and the change in the direction of development of cells for the sake of survival in a toxic environment and adaptation to these closed conditions was shown.

This dissertation is the first research work to study polymorphisms in different tissues of different wheat genotypes in Azerbaijan, where it was shown that retrotransposons move in somatic cells.

It was found that the mobility of retrotransposons was also different as a result of the differences in the microenvironment affecting different parts of the plant.

In the dissertation work, a phylogenetic tree of wheat genotypes was constructed for the first time and the genetic similarity coefficient between samples was determined.

The possibility of evaluating wheat genetic stock collections and confirming the genetic stability of varieties and species *in vitro* using IRAP (inter-retrotransposons amplified polymorphism) markers was confirmed.

It was recommended that deviations from the norm detected during micropropagation and such polymorphic varieties and species be included as initial material in various targeted sustainability selection studies.

The results of the dissertation work can be included in the teaching of plant physiology and biotechnology subjects in biological faculties of higher education institutions.

Publication, approval and application of the dissertation. Based on the materials of the dissertation, 24 scientific works were published in local and foreign publications, including 8 articles and 16 theses.

The main scientific results of the dissertation work were

presented at the scientific conference dedicated to the 90th anniversary of Academician Vahid Hajiyev (Baku, 2018), the scientific-practical conference of Young Researchers dedicated to the 90th anniversary of Academician Jalal Aliyev (Baku, 2018), the 2nd International Karabakh Congress of Applied Sciences "In Memory of Victory Day and Martyrs" (Baku, 2022), the 11th International Conference on Achievements and Difficulties in Biology (Baku, 2022), Materials of the International Scientific Conference on "Actual Problems of Modern Natural Sciences" (Ganja, 2023), the XXVIII International Scientific-Practical Conference "Methods of Unusual Development of Science and Thought" (Madrid, 2023), Biotic and Abiotic Stress Responses, Memory and Epigenomic Diversity in Plants (Tirana, 2024), "Epigenetic Mechanisms of Plant Adaptation to Climate Change" IV It was presented and discussed at the EPI-CATCH conference (Novi Sad, 2024), the VII Symposium on Eurasian Biodiversity (SEAB) (Erzurum, 2024), the Republican Scientific-Practical Conference "The Role of Universities in the Development of Innovative Ecosystems" (Ganja 2024), the XIV International Scientific-Practical Conference "Actual Problems of Humanity and Ways to Solve Them" (Munich, 2024), as well as at scientific seminars of the Institute of Molecular Biology and Biotechnologies of the Ministry of Science and Education of the Republic of Azerbaijan.

Organization where the dissertation work was carried out. The dissertation work was carried out at the Plant Biotechnology Laboratory of the Institute of Molecular Biology and Biotechnologies of the Ministry of Science and Education of the Republic of Azerbaijan and at the Plant Tissue Culture Laboratory of the Genetics Department, Faculty of Science, Yıldız Technical University, Istanbul, Turkey.

Structure and scope of the dissertation.

The total volume of the dissertation work consists of an introduction, 5 chapters, conclusion, results, recommendations, list of used literature, list of abbreviations and appendices. In the structure of the dissertation, the introduction consists of 12944 characters, the first chapter consists of 63,780 characters, the second chapter consists of 11,468 characters, the third chapter consists of 38,002 characters, the

fourth chapter consists of 15,926 characters, the fifth chapter consists of 34,459 characters, the conclusion, results consist of 9,658 characters, and recommendations consist of 396 characters. The total volume of the dissertation, excluding the list of literature, is 186,632 characters.

CHAPTER I
LITERATURE REVIEW

Section 1.1 of the dissertation provides extensive information on the biological properties of wheat plants, section 1.2 provides information on the concept of callus and its formation mechanisms, factors affecting the efficiency of callus culture, explant type, the effect of phytohormonal regulation on callus induction, and section 1.3 provides information on the application of callus culture in biotechnology and agriculture. In other sections, the effects of NaCl salt and heavy metal stress factors on morphogenesis processes, as well as epigenetic changes in plants and the role of retrotransposons in epigenetic processes are analyzed in detail.

CHAPTER II
RESEARCH MATERIAL AND METHODS

The research work used local wheat varieties Azamatli-95, Barakatli-95, Gobustan, Jumhuriyet-100, Gyrmyzy bugda and Nurlu-99 of the Scientific Research Institute of Agriculture of the Ministry of Agriculture of the Republic of Azerbaijan.

As explants, immature embryos of wheat grains at the end of the milk phase and at the beginning of the wax phase, measuring 1.3-1.8 mm, were used.

For callus induction, the explants isolated under aseptic conditions were planted in "*M-S nutrient medium*"[7] supplemented with 2,4-D.

[7] Murashige T, Skoog F (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15(3):473-497.

In the next stage of the research, the effect of phytohormones belonging to the auxin and cytokinin classes on callusogenesis, proliferation and callus mass accumulation was studied.

To achieve this goal, three variants of the nutrient medium were prepared, differing in hormonal composition, which allowed assessing the effect of the medium on the development of callus and its dynamics. In order to study the effect of different concentrations and combinations of phytohormones on the morphogenesis process in callus, the following variants were used:

- Variant I: M-S+3 mg/l 2,4-D
- Variant II: M-S+2 mg/l 2,4-D + 1 mg/l kinetin
- Variant III: M-S+1 mg/l IAA + 0.5 mg/l kinetin
- Variant IV: M-S+0.5 mg/l NAA + 1 mg/l BAP

After obtaining sufficient biomass during two subcultivation periods, the callus culture was transferred to selective nutrient media containing 100 mM, 150 mM, 200 mM and 250 mM NaCl salt concentrations. The callus culture was maintained in the dark.

In order to study the effect of heavy metals on callus proliferation, the callus mass collected after two subcultivations was transferred to a new nutrient medium supplemented with $CdSO_4$ salt (10 mg and 20 mg).

IAA (İndole Acetic Acid) was used to induce the root systems of regenerant plants. Cultivation was carried out under artificial climate conditions with a 16/8 hour photoperiod, 2000-4000 lux, 70% humidity, and a temperature of 24-26°C. Regenerant plants were adapted to the soil environment and leaf samples were collected for molecular analyses.

Molecular analyses were performed using the "*IRAP-PCR method* "[8] with primers specific for retrotransposons Sukkula, Houba and Sire1. The primers with the following sequences were used in the studies:

Sukkula " GGAACGTCG GCATCGGGCTG "

Houba-F " CTTCGAGTGGGCTAAGGCCC "

[8] Kidwell K. K., Osborn T. C. Simple plant DNA isolation procedures. Plant Genomes: Methods for Genetic and Physical Mapping, 1, 1992.

Houba-R " GTTTCGACCAAGCAGCCGGTC "
Sire1 " CAGTTATGCAAGTGGGATCAGCA "

The genetic similarity between the samples was assessed phylogenetically using the "*UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method* "[9] using the PAST program.

Polymorphism indices based on retrotransposon activity were statistically calculated using the Jaccard coefficient and the results were analyzed in three biological replicates, in a comparative manner with intact plants.

CHAPTER III
FACTORS AFFECTING WHEAT CALLUS PROCESSING

3.1. Effect of explant size on wheat callus formation

To obtain callus culture, immature wheat grain germs of different sizes (0.6-0.8 mm, 1.3-1.8 mm and 2.2-2.5 mm) taken at the end of the milk phase and the beginning of the wax phase were used as explants.

Table 1.

Callus induction in explants of different sizes (number of inoculated embryos, in %)

Genotypes	Explant size								
	I subcultivation			II subcultivation			III subcultivation		
	0,6-0,8 mm	1,3-1,8 mm	2,2-2,5 mm	0,6-0,8 mm	1,3-1,8 mm	2,2-2,5 mm	0,6-0,8 mm	1,3-1,8 mm	2,2-2,5 mm
Azamatli-95	**46%**	**88%**	**60%**	**68%**	**89%**	**71%**	**69%**	**93%**	**63%**
Barakatli-95	**58%**	**97%**	**55%**	**63%**	**99%**	**55%**	**63%**	**99%**	**63%**
Gobustan	**42%**	**70%**	**45%**	**54%**	**87%**	**47%**	**54%**	**92%**	**51%**
Jumhuriyet-100	**23%**	**65%**	**40%**	**48%**	**78%**	**52%**	**48%**	**78%**	**57%**
Gyrmyzy bugda	**49%**	**92%**	**50%**	**56%**	**95%**	**54%**	**56%**	**95%**	**54%**
Nurlu-99	**49%**	**89%**	**45%**	**58%**	**89%**	**60%**	**58%**	**89%**	**60%**

[9] Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica, 4, 9 p

Direct germination was more common in large-sized explants transferred to nutrient medium compared to explants of other sizes. Also, the germination rate of immature embryos increased proportionally with their size. In large-sized embryos, however, the callus formation process was very weak due to the strong tendency to differentiation.

3.2. Effect of phytohormones on callus formation in wheat explants

In the next stage of the study, the effect of 2,4-D phytohormone concentrations of 2 mg/l, 3 mg/l, 4 mg/l and 5 mg/l on callus induction in wheat explants was studied (Figure 1).

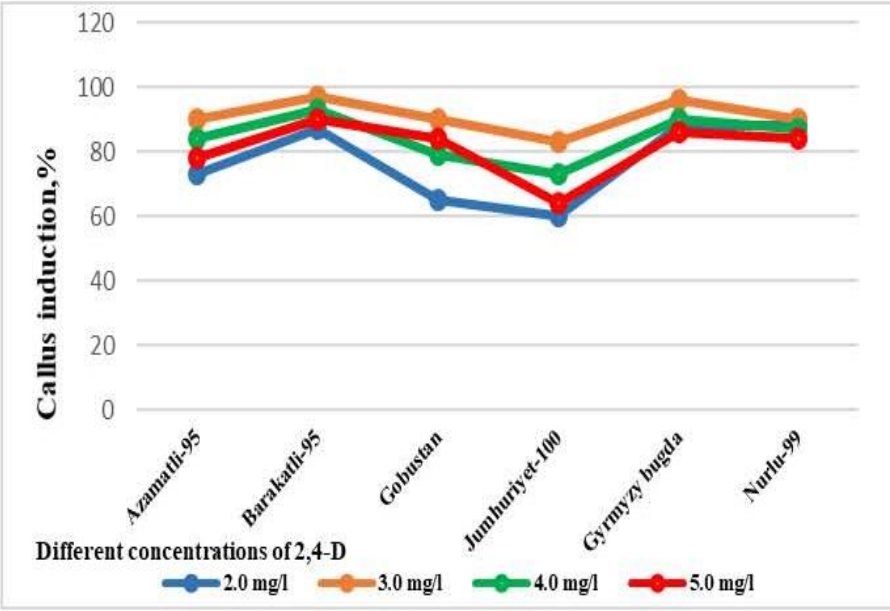

Figure 1. Effect of different concentrations of 2,4-D on callus induction

The most optimal concentration was 3 mg/l. At this concentration, a callus mass with a homogeneous structure and living cells was obtained. At a concentration of 2 mg/l, callus

formation was weak, and at concentrations of 4 mg/l and 5 mg/l, negative changes in the morphology of the callus occurred.

In addition to 2,4-D, the effect of other phytohormones and their various combinations on the callusogenesis process was studied (Table 2).

Table 2.

Effect of different phytohormone combinations on callus biomass fresh weight (g)

Genotype	2,4 D (3 mg L^{-1})		2,4 D (2 mg L^{-1}) + Kinetin (1mg L^{-1})		IAA (1 mg L^{-1}) + Kinetin (0,5 mg L^{-1})		NAA (0,5 mg L^{-1}) + BAP (1 mg L^{-1})	
	Subcultivation							
	II	III	II	III	II	III	II	III
Azamatli-95	4.80 ± 0,03	7.20 ± 0,03	3,81 ± 0,02	5,72 ± 0.03	2,80 ± 0,03	3,53 ± 0,04	3,12 ± 0.02	4,93 ± 0.03
Barakatli-95	5.61 ± 0,01	9.43 ± 0,01	5,00 ± 0,03	7,80 ± 0.03	3,33 ± 0,02	5,03 ± 0.02	3,83 ± 0,03	6,14 ± 0,01
Gobustan	4.13 ± 0,03	7.32 ± 0,02	3,42 ± 0,02	5,63 ± 0.03	2,94 ± 0.04	4,04 ± 0,03	3,71 ± 0,01	5.63 ± 0.02
Jumhuriyet-100	3.03 ± 0,03	5.52 ± 0,01	2,70 ± 0,04	4,53 ± 0,04	2,01 ± 0,03	3,03 ± 0,04	2,80 ± 0,03	3.44 ± 0,02
Gyrmyzy buğda	6.02 ± 0,03	8.90 ± 0,04	5,84 ± 0,01	7,84 ± 0,02	4,23 ± 0,03	5,44 ± 0.02	5,61 ± 0,02	7,14 ± 0,01
Nurlu-99	5.92 ± 0,03	7.31 ± 0,03	4.21 ± 0,02	5.32 ± 0,04	3,23 ± 0,02	4.13 ± 0,04	3,94 ± 0,03	4,93 ± 0.01

The highest callus biomass and proliferation intensity were observed in the nutrient medium containing 3 mg/l 2,4-D (variant I). Cytokinins - kinetin and BAP in some cases weakened the proliferation process, but contributed to the increase in morphogenic ability. The callusogenesis process was weaker in the IAA and NAA-containing media, but the formation of morphogenic calluses was recorded in certain genotypes in variants III and IV. The Barakatli-95 and Gyrmyzy bugda genotypes differed from other genotypes in terms of callus induction, proliferation intensity and biomass accumulation dynamics in all tested nutrient medium variants. In particular, the highest results were obtained in the III

subculturing stage of the 2,4-D (3 mg/L) test variant - 9.43 g).

3.3. The effect of genotype on wheat callus formation

Of the studied varieties, the highest callus induction frequency was characteristic of the Barakatli-95 and Gyrmyzy bugda genotypes. The concentration of 2,4-D phytohormone at 3 mg/l had the best effect on biomass growth. Although callus proliferation was high in some genotypes, morphogenesis was observed to be weak (e.g., Azamatli-95). Meristematic areas were formed in morphogenic calluses (Figure 2).

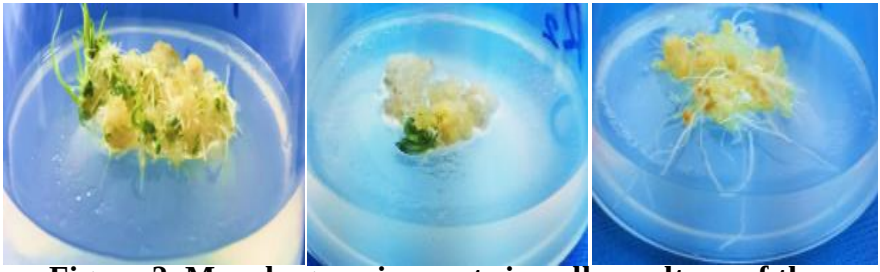

Figure 2. Morphogenesis events in callus culture of the Nurlu-99 genotype: hemorrhizogenesis, hemmogenesis and rhizogenesis

Gyrmyzy bugda and Nurlu-99 genotypes showed high morphogenic potential. Although the process started late in the Jumhuriyet-100 variety, clearly distinguishable embryoids measuring 0.5-0.8 cm were formed after 10 days, and the formed regenerants developed healthily.

As mentioned above, although the callus culture of the Azamatli-95 genotype had an intensive proliferation capacity, its morphogenetic potential was extremely weak. Such dissociation of proliferation and morphogenesis indicates the presence of genotype-specific limitations in the implementation of differentiation programs at the cellular level. In contrast, the Gobustan variety, despite its weak callus induction capacity, was able to maintain its morphogenetic potential during long-term *in vitro* cultivation. This may indicate the existence of mechanisms

that ensure the stabilization of differentiation pathways that are not directly related to cell division activity.

The observations show that genetic and physiological-biochemical regulation of morphogenesis in different wheat genotypes is carried out in different ways, and this involves the participation of both nuclear genes and epigenetic factors at different levels. The regenerant plants obtained as a result of the studies prove that even if callus formation is limited, the restoration of the morphogenetic program is possible under the conditions of optimization of the appropriate genotypic fund and cultivation conditions.

The next stage of cultivation is the transfer of regenerants with sufficiently strong roots to an artificial soil substrate. Adaptation of regenerant plants was carried out for 2-3 weeks at room temperature. 0.5 mg/l of IAA solution was added to the soil for strengthening the roots in the artificial soil environment. Regenerant plants adapted in the artificial substrate were transferred to the natural planting area for further development. At this time, their size was between 12-15 cm. Significant growth was observed in regenerant plants within 3 months. At the final stage of the study, the process of strengthening and rooting in the soil was successfully carried out (Figure 3).

Figure 3. Transfer of regenerant plants to the planting field

CHAPTER IV
THE EFFECT OF ABIOTIC STRESS FACTORS ON WHEAT CALLUS DEVELOPMENT

4.1 The effect of salt stress on wheat callus development

To create stress conditions in callus culture, 100 mM, 150 mM, 200 mM and 250 mM NaCl salt were added to the culture medium.

At the end of the II subcultivation, 2 g of callus mass was taken from each genotype sample and transferred to a new selective culture medium, and the biomass increase was evaluated at the end of the III subcultivation (Table 3).

Table 3.

Callus biomass growth at the end of III subcultivation (g)

Genotype	Control	100 mM NaCl	150 mM NaCl	200 mM NaCl	250 mM NaCl
Azamatli-95	**4.80**±0,03	**3.82**±0,03	**3.33**±0,02	**2.64**±0,04	**1.12**±0,03
Barakatli-95	**5.41**±0,01	**4.51**±0,02	**4.01**±0,01	**3.02**±0,02	**1.43**±0,04
Gobustan	**5.01**±0,02	**4.20**±0,04	**3.23**±0,03	**2.53**±0,03	**1.21**±0,04
Jumhuriyet-100	**3.91**±0,01	**3.23**±0,03	**2.54**±0,04	**2.24**±0,03	**1.01**±0,01
Gyrmyzy bugda	**5.33**±0,04	**4.24**±0,01	**3.51**±0,03	**3.11**±0,02	**1.13**±0,01
Nurlu-99	**4.52**±0,03	**4.02**±0,03	**3.40**±0,02	**2.41**±0,04	**1.15**±0,02

As can be seen from the table, the best results were shown by the genotypes Barakatli-95,Gyrmyzy bugda and Gobustan at all concentrations of NaCl.

During the study, the increase in callus mass at 150 mM and 200 mM concentrations of NaCl was less than at 100 mM concentration (Figure 4).

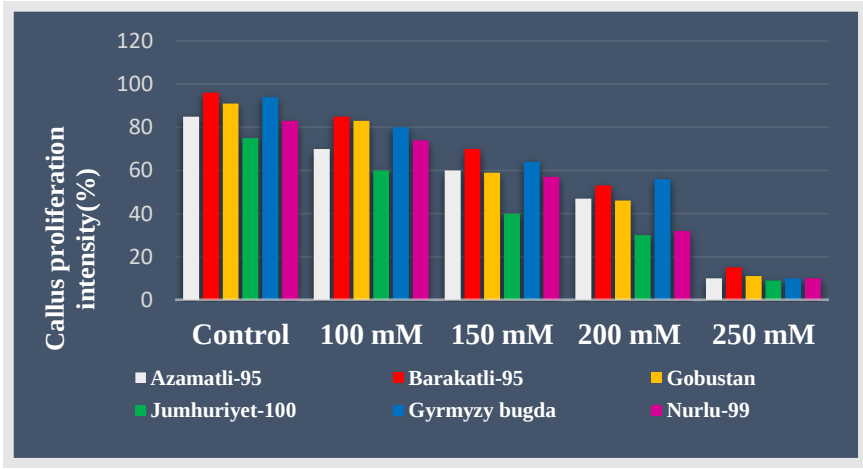

Figure 4. Growth of callus mass at the end of the third subcultivation. In %.

At a concentration of 100 mM NaCl, the highest results in the growth of callus biomass were shown by the Gyrmyzy bugda and Barakatli-95 genotypes, and the weakest results were shown by the Jumhuriyet-100 genotype.

At a concentration of 150 mM NaCl, the positive responses of the Barakatli-95 and Gyrmyzy bugda durum wheat genotypes to the stressor were recorded, and an increase in callus biomass was observed. According to this indicator, the weakest result was recorded in the Jumhuriyet-100 genotype.

At a concentration of 200 mM, the growth of callus in wheat genotypes was weak, and as a result, very weak morphogenic areas were formed.

During subculturing with a sublethal dose of the selective agent at a concentration of 250 mM, the cultivation of callus resulted in necrosis in all genotypes.

The formation of morphogenic areas in calluses in the wheat genotypes Jumhuriyet-100, Gyrmyzy bugda, and Nurlu-99 at a concentration of 150 mM NaCl only indicates that they retain their regeneration ability and can be used as potential donor material in breeding programs.

4.2. Effect of heavy metal stress on wheat callus development

The effect of cadmium (Cd^{2+}) on the development of wheat callus culture was systematically studied in the study. Experiments conducted with 10 mg/l and 20 mg/l concentrations of $CdSO_4$ salt showed that callus did not form as a result of direct placement of explants in cadmium-containing medium. After two stress-free subcultivations, proliferation was observed when calluses were transferred to $CdSO_4$-containing medium. At a concentration of 10 mg/l, in some genotypes, including Barakatli-95 and Gyrmyzy bugda, despite the formation of amorphous and watery callus, no cell death was observed. At a concentration of 20 mg/l, the structure of the callus tissue was more compact and finely divided.

At concentrations of 10 mg/l and 20 mg/l of the selective agent, the best results were observed in the genotypes Barakatli-95,Gyrmyzy bugda and Gobustan, intermediate results were observed in the genotypes Nurlu-99 and the weakest proliferation was observed in the genotype Jumhuriyet-100 (Figure 5).

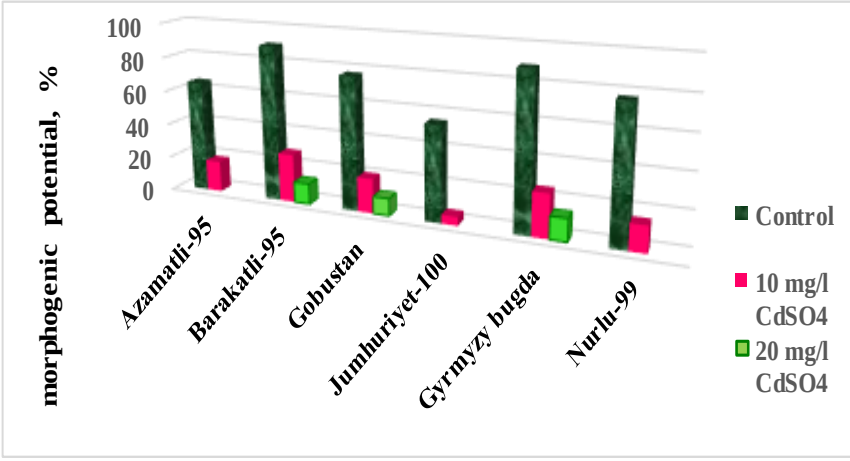

Figure 5. Effect of $CdSO_4$ salt on the morphogenetic potential of callus depending on genotype.

In variant 1, callus cultures obtained from Nurlu-99, Barakatli-95 and Gyrmyzy bugda genotypes were transferred to appropriate nutrient medium for morphogenesis induction and plant regeneration. Morphogenic zones were recorded only in the callus tissue of the Barakatli-95 genotype, and green pigmented zones and embryo-like formations prevailing in the light regime were observed. Although long-term cultivation was carried out, no plants were obtained.

CHAPTER V
STUDY OF EPIGENETIC CHANGES IN WHEAT PLANTS *IN VIVO* AND *IN VITRO*

5.1. Effect of NaCl salt stress on the mobility of Houba, Sukkula and Sire1 retrotransposons *in vivo* and *in vitro*

A high level of activity of Houba retrotransposons was observed especially in callus samples under the influence of 150 mM and 200 mM NaCl salt stress (86-100%). Although the Jumhuriyet-100 genotype showed high polymorphism in the callus stage, the level of polymorphism was low in the leaf samples of the mother plant (33-50%).

A high level of activity of Sukkula retrotransposon was observed in callus and leaf samples in the Nurlu-99 and Gyrmyzy bugda genotypes. In the Jumhuriyet-100 genotype, polymorphism was mainly observed in callus on day 15 (0-67%), and on day 30, no polymorphism was recorded at all (0%).

The Sire1 retrotransposon also had high activity in callus and leaf samples of the Gyrmyzy bugda and Nurlu-99 genotypes. In the Jumhuriyet-100 genotype, polymorphism in callus was more limited, but genetic diversity in leaf samples was recorded in a wider range (83-100%).

Callus and leaf samples exposed to 150 mM and 200 mM NaCl stress *in vitro* were grouped into different genetic clusters based on the Jaccard similarity coefficient (Figure 6). The highest polymorphism and similarity differences were observed between callus samples. Thus, it was shown that retrotransposon activity

plays an important role in the formation of the genetic response to salt stress.

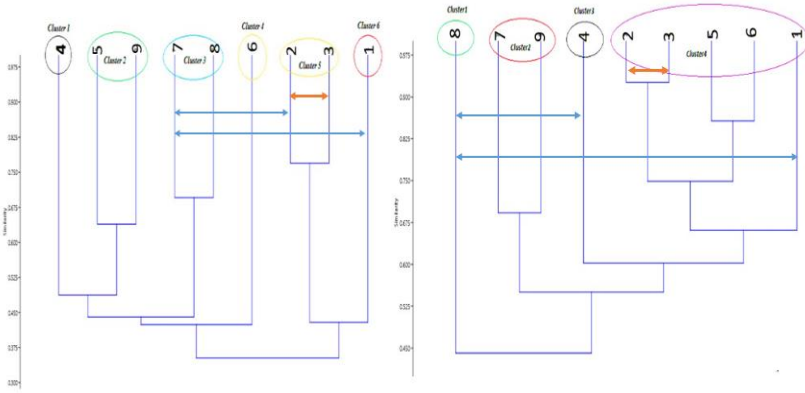

Figure 6. Dendrogram showing the relationship between *in vitro* callus and leaf samples generated with Houba, Sukkula and Sire1 primers based on UPGMA cluster tree analysis. Jumhuriyet-100 (1-control, 2-150mM NaCl, 3- 200mM NaCl) ;Gyrmyzy bugda (4- control, 5-150mM NaCl, 6- 200mM NaCl); Nurlu-99 (7- control, 8-150mM NaCl, 9- 200mM NaCl)

5.2. Effect of $CdSO_4$ salt stress on the mobility of Houba, Sukkula and Sire1 retrotransposons under *in vivo* and *in vitro* conditions

Under the influence of $CdSO_4$ stress, the activity of Houba retrotransposons was mainly observed in callus samples, and no polymorphism was recorded in intact leaves.

The activity of Sukkula retrotransposon against heavy metal stress differed depending on the genotype, tissue type and stress duration. The Jumhuriyet-100 genotype showed high activity in callus and leaf samples, while in Nurlu-99, the mobility of Sukkula retrotransposon increased significantly, especially on day 30. In Gyrmyzy bugda, the activity of Sukkula retrotransposon increased mainly in later stages and at high cadmium concentrations.

Polymorphism in Sire1 retrotransposon was observed only in leaf samples, and no activity was recorded in callus samples. The highest activity indicators were observed in the Jumhuriyet-100 and Nurlu-99 genotypes on day 30.

During phylogenetic analyses, samples belonging to the same wheat genotypes were grouped in the same or close clusters with the highest genetic similarity coefficient (Figure 7).

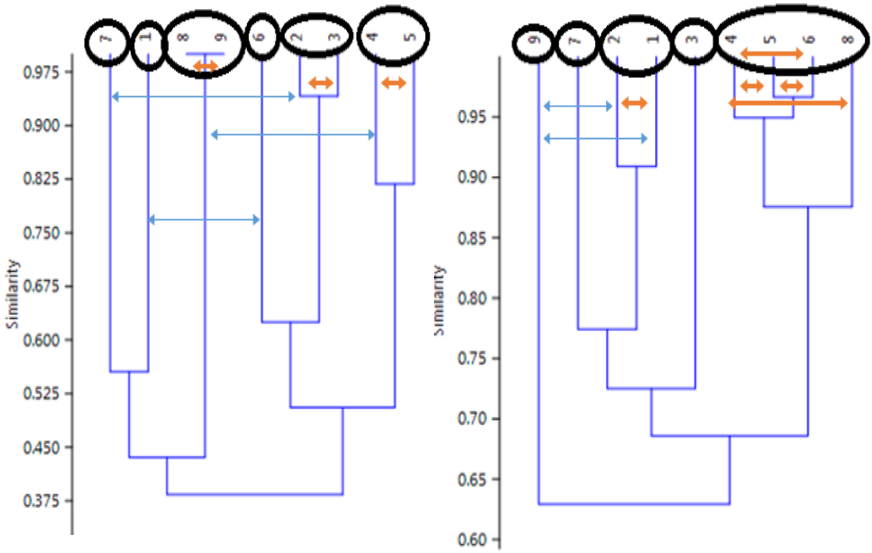

Figure 7. Dendrogram generated based on retrotransposons in callus (left) and leaf (right) samples based on UPGMA cluster tree analysis. Jumhuriyet-100 (1-control, 2- 10 mg/l $CdSO_4$, 3-20 mg/l $CdSO_4$), Gyrmyzy bugda (4-control, 5- 10 mg/l $CdSO_4$, 6-20 mg/l $CdSO_4$), Nurlu-99 (7-control, 8- 10 mg/l $CdSO_4$, 9-20 mg/l $CdSO_4$).

The highest similarity was observed between callus samples of Nurlu-99 and Jumhuriyet-100 wheat genotypes, as well as leaf samples of Gyrmyzy bugda. The lowest similarity coefficient was recorded between different genotypes, confirming their genetic distance.

5.3. Mobility of retrotransposons in regenerant plants

In the analyses conducted based on the Jaccard similarity coefficient, the activity of retrotransposons and genetic diversity changed over time.

On the 15th day, the highest activity of Houba retrotransposons was observed in Gyrmyzy bugda and Nurlu-99 wheat calli, but no polymorphism was recorded in Jumhuriyet-100 calli. No activity was recorded in regenerant plants on the 30th day of the experiment.

On the 15th day, the activity of Sukkula retrotransposons was observed at the highest level in the Gyrmyzy bugda genotype (0-67%), in Nurlu-99 it was in the range of 0-56%, and in Jumhuriyet-100 a polymorphism value was recorded in the range of 0-20%. On the 30th day, the highest polymorphism indicators (0-70%) were observed in Nurlu-99, and lower levels were recorded in Jumhuriyet-100 and Gyrmyzy bugda.

According to the results of the 15th day of Sire1 retrotransposons, the highest activity (0-50%) was recorded in the Gyrmyzy bugda and Nurlu-99 genotypes, while no mobility was recorded in Jumhuriyet-100. On the 30th day, the Sire1 retrotransposons in the Nurlu-99 variety showed the highest activity (0-73%), while the lowest result was recorded in Jumhuriyet-100 (0-13%). In the samples of the callus Nurlu-99 genotype, the Sire1 retrotransposon demonstrated higher mobility (0-83%). The polymorphism indicators in the regenerants were recorded at the highest in Nurlu-99 (0-58%), and the lowest in Jumhuriyet-100 (0-9%).

As can be seen from the figure, the control, callus and regenerant plant samples of the wheat genotypes were grouped into 6 main clusters based on the Jaccard similarity coefficient (Figure 8). Individual samples located in clusters 1, 4, 5, and 6 showed that each was genetically distinct from the other samples.

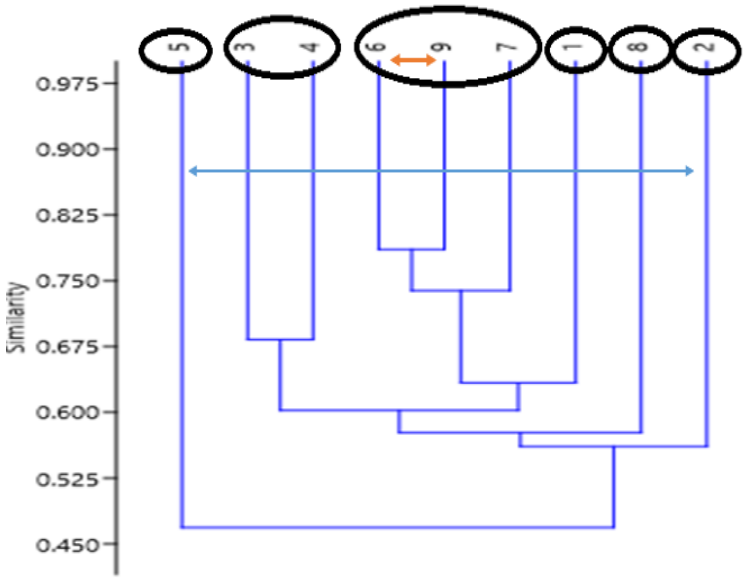

Figure 8. Dendrogram generated by UPGMA cluster tree analysis based on 3 primers (Houba, Sukkula and Sire 1). Control (1-Jumhuriyet-100, 2-Gyrmyzy bugda, 3-Nurlu-99), callus (4-Jumhuriyet-100, 5- Gyrmyzy bugda, 6-Nurlu-99), regenerant (7-Jumhuriyet-100, 8- Gyrmyzy bugda, 9-Nurlu-99).

FINAL ANALYSIS OF THE RESULTS OBTAINED IN THE RESEARCH

Regulation of the activity of retrotransposons plays a crucial role in directing important indicators such as genetic stability and variability in plants and allows for the formation of targeted genetic diversity in the selection process.

Within the framework of the dissertation, for the first time, the epigenetic activity of retrotransposons in wheat genotypes was studied in Azerbaijan, and the interaction of these processes with *in vitro* regeneration and genetic stability was scientifically substantiated. The study showed that not only genetic but also epigenetic factors - especially the mobility of retrotransposons - are of great importance in the adaptation of plants to abiotic stresses.

Assessment of the degree of genetic diversity and stability among wheat varieties using IRAP markers is proposed as an effective method for both breeding programs and genetic resource management.

In addition, the effect of phytohormone balance on morphogenesis in artificial nutrient media and the role of stress factors in epigenetic regulation were comprehensively investigated. The genetic and physiological characteristics observed in the callus culture of the Gobustan variety indicate that this genotype has a high morphogenic potential and allows it to be used as a donor in obtaining regenerative plants. The research results create a powerful platform for reliable assessment of genetic stability in *in vitro* selection and micropropagation processes. At the same time, the application of retrotransposons as genetic markers ensures operational and efficient identification of samples to be included in the selection.

The results of this scientific work can be used not only for agricultural selection but also as a rich and relevant source in teaching such subjects as biotechnology and plant physiology in higher education institutions.

CONCLUSIONS

1. Comparative analysis of callus formation processes showed that the optimal size of the embryo taken as an explant was 1.3-1.8 mm. In this case, the cells have high metabolic activity, plasticity, active division ability and provide the most effective morphogenic callus formation. Although small embryos have some regeneration potential, they are physiologically limited in their development. Large embryos, on the other hand, reach a level of relative autonomy and, despite the formation of callus *in vitro*, without dependence on either phytohormonal or other physiological factors of the environment, can germinate directly and give rise to a normal plant in all respects [1, 4, 7, 13, 23].

2. The Barakatli-95 and Azamatli-95 genotypes demonstrated high-frequency callus induction, but the varieties differed significantly in terms of morphogenesis and regeneration ability. This indicates the existence of genetic and physiological mechanisms controlling phenotypic traits. It is believed that the observed differences in the generation of competent cells and the ability to regenerate *in vitro* are due to genetic determination affecting the active expression of key genes regulating cell proliferation and differentiation [1, 4, 5, 14, 19, 21, 22, 24].

3. The low frequency of callus formation of the Gobustan genotype and the preservation of its high morphogenetic potential in long-term subcultivations (6 months) indicate its genetic and physiological resistance to *in vitro* cultivation conditions. The "genotype" factor, giving maximum impetus to the development stage of morphogenic callus *in vitro* conditions, keeps the process under control until the end, which significantly affects the formation of cell cultures and regenerative plants. The uniqueness of the genetic and physiological parameters of the Gobustan callus culture can be used in cell selection programs for the mass production of regenerative plants, as well as as a donor of a high morphogenesis trait [2, 3, 4, 19].

4. During the cultivation of immature wheat germs, a negative correlation was established between the proliferation intensity of increasing NaCl concentration in the selective system and the dynamics of callus biomass accumulation. It was proven that there is no direct relationship between the processes of morphogenesis and regeneration in both control and selective conditions. Under simulated salinity conditions, only callus cultures of the Jumhuriyet-100, Gyrmyzy bugda and Nurlu-99 varieties were capable of plant reconstruction at a concentration of 150 mM NaCl salt. This can be explained by the presence of competent cells that maintain totipotency in the callus masses [4, 12, 18, 20].

5. General regularities of wheat callus tissue development when using $CdSO_4$ as a stress factor were determined. The

nonlinear nature of the relationship between the intensity of morphogenesis and selective stress was shown. At a concentration of 10 mg/l of $CdSO_4$ salt, the share of morphogenic callus in the total mass was between 5 and 28%, and at a concentration of 20 mg/l, it was between 0 and 13%. It was determined that in a selective medium with a concentration of 20 mg/l, the callus tissue of the Jumhuriyet-100, Nurlu-99 and Azamatli-95 genotypes completely lost their morphogenetic ability [10, 12, 16].

6. For the first time in Azerbaijan, the rice-specific Houba, barley-specific Sukkula and soybean-specific Sire1 retrotransposons were detected in Jumhuriyet-100, Gyrmyzy bugda and Nurlu-99 local wheat varieties using the IRAP marker method. These findings may play an important role in studying the genetic diversity of local wheat varieties and their interactions with various plant species. The detection of the mentioned retrotransposons in the corresponding plant species indicates that various transition events (HT-horizontal transfer) occurred in the genetic composition of these species and that the genetic information of different plant species was combined. This phenomenon can be explained by the idea that mobile elements could be included in the genome sequence during the evolution of the plant during the domestication of wheat [6, 8, 9, 11, 17, 20].

7. For the first time, the movement activity of barley (Sukkula), rice (Houba) and soybean (Sire1) retrotransposons in callus and leaves under the influence of salt stress in wheat plants was studied using the IRAP marker method. The activation of retrotransposons caused epigenetic changes indicating the level of adaptation of the plant to abiotic stresses. *In vitro* conditions, compared to *in vivo*, enhanced the activity of retrotransposons, which is reflected in the emergence of genotypic and phenotypic diversity in plants [8, 9, 12, 15, 20].

8. Phylogenetic analysis revealed that retrotransposons exhibited higher activity in wheat callus culture under *in vitro* conditions. This suggests that complex stress, both under

specific *in vitro* conditions and due to the characteristics of the selective nutrient medium, plays a significant role in the activity of retrotransposons in callus [9, 15, 20].

PRACTICAL RECOMMENDATIONS

1. The uniqueness of the genetic and physiological parameters of the Gobustan callus culture can be used in cell selection programs for the mass production of regenerative plants, as well as a donor of a high morphogenesis trait.

2. Based on the results of the dissertation work, the use of retrotransposons as genetic markers for efficient and rapid identification of samples included in the selection is recommended.

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The dissertation defense will be held on 10 september 2025 at 11.00 at the meeting of the BFD 1.07/1 Dissertation Council operating under the Microbiology Institute of AR ETN.

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The dissertation can be viewed in the library of the Microbiology Institute of AR ETN.

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