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Optimization *in vitro* cultivation conditions for an endemic species of Regel's pear

The decline in plant diversity is a pressing global issue driven by climate change, plant diseases, and human activities. This reduction in biodiversity poses significant threats to food security and the sustainability of ecosystems. Conserving wild plant species is crucial as they harbor genes that confer resistance to various biotic and abiotic stresses. One such important species is the Regel pear, endemic to Kazakhstan, known for its resistance to drought and diseases. During research efforts, samples of Regel pear were collected from their natural habitats. Using SSR markers, researchers identified samples that exhibited resistance to scab. In a pioneering step, optimal *in vitro* cultivation conditions were developed to preserve this economically valuable species. The sterilization process for establishing *in vitro* culture and subsequent regeneration was established. The multiplication conditions were refined using DKW nutrient medium supplemented with 1.0 mg/l BAP, 0.5 mg/l IBA, and 0.2 mg/l GA3. These measures aim to ensure the long-term conservation of genetic diversity and enhance agricultural resilience to environmental changes.

Keywords: Pyrus regelii, *invitro* culture, endemic, micropropagation.

Introduction

Declining biodiversity has a negative impact on food security, leading to the deterioration of ecosystems. According to the report on the state of plant genetic resources, several factors contribute to the decline in biodiversity, including climate and habitat changes, invasive species, and the over use of plant resources beyond their natural restoration levels [1, 2, 3].

Kazakhstan holds a leading position in plant diversity within Central Asia, boasting approximately 5,700 species of higher plants, of which 14 % are endemic. However, like many regions worldwide, Kazakhstan faces biodiversity loss due to anthropogenic influences, soil degradation, deforestation, and other factors [4]. The use of biotechnological methods is effective for preserving biodiversity and promoting sustainable development [5, 6, 7].

Kazakhstan is the homeland of many nut and fruit plant species, which are now cultivated globally [8, 9, 10]. According to Vavilov's centers of origin of cultivated plants, the Central Asian center includes species such as apple trees, pears, cherries, plums, pistachios, grapes, and others [11]. Pear species, represented by a wide variety of wild and cultivated local varieties, have significant importance in this region. Central Asia is a key center for the speciation of the genus *Pyrus* L. [12].

Pear production ranks second worldwide after apples. Pears are susceptible to various diseases, with some of the most dangerous being fire blight and scab. Scab is caused by fungi of the genus *Venturia*, specifically *V. naschicola* infecting Asian pear species and *V. pirina* affecting common pears [13, 14].

Wild pear species of fer allelic diversity and combinations that provide resistance and tolerance to various abiotic and biotic stresses. Many wild pear species, including *P. ussuriensis, P. pashia, P. korshinskyi, P. syriaca, P. hopiensis, P. gharbiana, P. betulifolia, P. calleryana, P. cossonii, P. dimorphophylla, P. fauriei, P. pyrifolia, P. ussuriensis, P. regelii, P. communis,* and *P. xerophila*, possess straits of drought, cold, and disease resistance [15, 16, 17].

Regel's pear *(Pyrus regelii)* is an endemic species growing in the Tien Shan and Pamir-Altai regions, with its habitat gradually shrinking. It grows singly or in groups on dry rocky slopes and rocks, among shrubs, in xerophytic woodlands, and thermophilic juniper forests [18]. In southern Kazakhstan, the Regel pear grows, recognized as an economically valuable species due to its significant drought resistance. This species can be utilized for a forestation of arid areas with poor soil conditions. Its fruits are very tart and astringent but can be used as drought-resistant roots tocks [19].

The purpose of this study is to optimize the cultivation conditions of the endemic Regel pear species, which is resistant to scab, in *in vitro* culture to preserve biodiversity and enhance sustainable development in Kazakhstan.

Experimental

The object of research was plant material of the Regel pear, which was collected in the Sairam-Ugam State National Natural Park, Tyulkubas branch (Fig. 1). Table 1 presents the coordinates of the selected samples.

A) specimen 1 B) specimen 2 C) specimen 3

Figure 1. Regel's pear in its natural habitat

Table 1

Coordinates of Regel's pear growth

Species	Number	Longitude	Latitude	Height above sea level, m
Regel's pear	specimen 1	E070°15.33'.	N42°40.33'	880
	Specimen 2	E70°15.091'	N42°41.345'	917
	Specimen 3	E70°15.645'	N42°40.789'	864

DNA extraction

DNA extraction from plant material was carried out according to the CTAB protocol. CTAB lysis buffer contained 2 % cetyltrimethylammonium bromide, 20 mM EDTA, 100 mM Tris-HCl, 1.4 mM NaCl, and 1 % PVP. Purification with chloroform was carried out twice.

Isolation protocol: Leaves were homogenized in 500 µl CTAB buffer for 3 min. at 30 Hz using a high-speed homogenizer Tissuelyser II Qiagen. After incubation at 65 °C for 60 minutes, samples were centrifuged at 20,000 rpm for 10 minutes, then the supernatant was extracted with an equal volume of chloroform and centrifuged for 15 minutes at 20,000 rpm. The extraction procedure was repeated twice. DNA was precipitated with 2/3 isopropanol and centrifuged at 14,000 rpm for 30 minutes. The precipitate was washed twice with 70 % ethanol, dried and dissolved in 100 µl of TE buffer [20].

Molecular genetic analysis

To study the genetic potential of pear resistance to pathogens, molecular genetic analysis was carried out. To select resistant genotypes to scab (*Venturia nashicola*), the SSR marker TsuENH101 (AB621905) was used. Sequence: F: TGCCTAATGGAAGGGTCCTA R: CAAGGAAGAGAAGACCGACG [21]. The 25 µl PCR reaction mixture contained the following: 5 µl template DNA (50 ng/µl); 3 µl PCR buffer (10x); 1.5 µl $MgCl_2$ (25 mM); 1.5 µl off or ward and reverse primers (10 pmol); 0.3 µl of Taq polymerase and 1.8 µl of dNTP (2 mM), the rest of the mixture was made up with deionized distilled water. Amplification

was performed using a 96-well thermal cycler (Applied Biosystems) with the following conditions: 95 °C for 2 min; 40 cycles 95 °C 30 sec, 55 °C 30 sec, 72 °C 45 sec; 72ºC 5 min.

Sterilization and establishment in vitro culture

To sterilize annual axillary buds of the Regel pear, the effects of different concentrations of hydrogen peroxide (H_2O_2) were studied. The following concentrations were tested: I — 3 % H_2O_2; II — 6 % H_2O_2; III — 9 % H_2O_2; IV — 12 % H_2O_2, in all cases the exposure time was 5 minutes.

The effect of nutrient media with the addition of the cytokinin 6-benzylaminopurine (BAP) at a concentration of 1.0 mg/l on the formation of the main shoot was studied. The following options were studied: I — MS with the addition of BAP — 1.0 mg/l; II — DKW with the addition of BAP — 1.0 mg/l; III — QL with the addition of BAP — 1.0 mg/l. In each variant, 30 explants were cultured.

Multiplicaton stage

To multiply shoots, we studied various combinations and concentrations of growth regulators BAP, gibberyllic acid (GA3), and indolyl-3-butyric acid (IBA) on the DKW nutrient medium. Treatments: I — DKW without growth regulators; II — DKW with BAP 0.1 mg/l, IBA 0.1 mg/l, GA 0.2 mg/l; III — DKW with BAP 0.5 mg/l, IBA 0.1 mg/l, GA 0.2 mg/l; IV — DKW with BAP 1.0 mg/l, IBA 0.1 mg/l, GA 0.2 mg/l; V — DKW with BAP 0.1 mg/l, IBA 0.5 mg/l, GA 0.2 mg/l; VI — DKW with BAP 0.5 mg/l, IBA 0.5 mg/l, GA 0.2 mg/l; VII — DKW with BAP 1.0 mg/l, IBA 0.5 mg/l, GA 0.2 mg/l; VIII — DKW with BAP 0.1 mg/l, GA 0.2 mg/l; IX — DKW with BAP 0.5 mg/l, GA 0.2 mg/l; X — DKW with BAP 1.0 mg/l, GA 0.2 mg/l.

In each treatment, 30 shoots were used.

Statistical processing

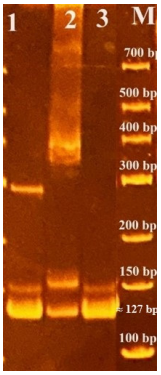
The experimental results were analyzed using one-way and two-way ANOVA, and significant differences were screened using Tukey's post hoc test. The results were analyzed in the statistical package SPSS 23.0 (IBM Inc., New York, USA). Data are expressed as means ± standard error.

Results and Discussion

The habitat of the Regel pear is declining due to diseases, climate change, anthropogenic impact, insufficient watering, and other factors [22]. Scab, caused by two species of fungi, *Venturia pirina* and *Venturia nashicola*, is one of the most severe and common diseases affecting European and Asian pears. Scab pathogens impact the leaves, fruits, and branches of pears. Scab resistance is a crucial goal in pear breeding, along with improving fruit quality, yield, and storage ability. Despite the development of a few scab-resistant pear varieties, none are produced in large quantities [23].

Several studies have focused on mapping the pear genome to identify scab resistance genes, one of which is Vnk. Based on this gene; molecular markers such as SSR, AFLP, and RAPD have been developed [24]. SSR markers, in particular, were developed to determine resistance to black spot [25].

To select scab-resistant samples of Regel pear, molecular genetic analysis was conducted using a microsatellite marker. In the first stage, DNA of the required quality and quantity was extracted from pear leaves. DNA concentration was measured using a small volume spectrophotometer. PCR was carried out with the SSR marker TsuENH101 to select scab-resistant samples. Detection was performed in 8 % polyacrylamide gel (Fig. 2).



M — molecular weight marker
(Ferment as, 25-700 bp);
1 — specimen 1;
2 — specimen 2;
3 — specimen 3

Figure 2.
Electropherogram of Regel pear using
the TsuENH101 SSR marker

According to the results of the analysis, it was shown that all samples tested with the SSR marker Tsu ENH101 were resistant to *Venturia nashicola*. A polymorphic allele with a length of 127 bp was amplified, which demonstrated a significant connection with the Vnk gene responsible for scab resistance (*Venturia nashicola*). Consequently, a study of three Regel pear trees confirmed their resistance to scab. These trees were then established *in vitro* culture for further micropropagation.

One of the critical stages of micropropagation is the sterilization nofex plants for establishment *in vitro* culture. Hydrogen peroxide (H_2O_2) was used as the primary sterilizing agent, a common practice in micropropagation of various plant species [26, 27, 28, 29].

Sterilization of explants was performed in two stages. In the first stage, one-year-old axillary buds were washed multiple times in a soap solution on a magnetic stir restore move surface dust and dirt. Then, under aseptic conditions, they were treated with hydrogen peroxide according to the concentrations specified in the research methods. The results were analyzed on the 14th day of cultivation. Sterilization was considered most effective when the explants remained green and showed no signs of infection (bacterial or fungal) or tissue necrosis.

The study revealed that the most optimal sterilization for Regel pear involved treating the explants with 12 % H_2O_2 for 5 minutes. This method resulted in 70 % of the explants being viable (Fig. 3).

Other studies on pear sterilization have used various sterilizing agents. For example, sodium hypochlorite was found effective for the Pyro dwarf rootstock of the species *P. communis* L. [30]. Additionally, 4 % $HgCl_2$ has been used in several works for the sterilization of common pear and Syrian pear [31, 32]. Czech researchers used 0.15 % $HgCl_2$ in their work on the micropropagation of pear [33]. Therefore, it is necessary to select an appropriate sterilizing agent for each specific type and variety of pear.

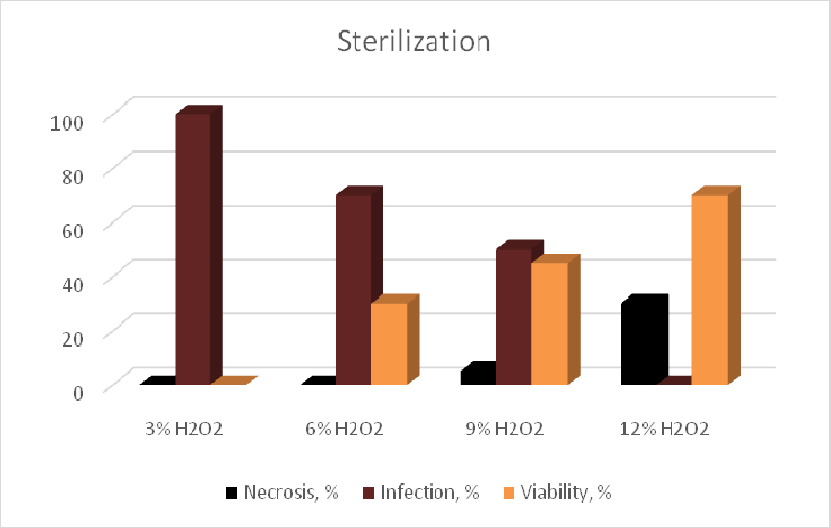
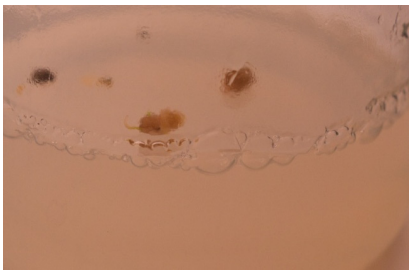


Figure 3. Results of sterilization of axillary buds of the Regel pear

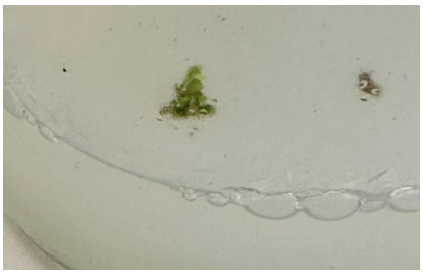
The next step after selecting the optimal sterilization regime is the formation of the main shoot. For micropropagation, the main shoot must be produced by direct organogenesis, without evidence of callus formation, as somaclonal variation may occur.

We studied several variants of culture media with the addition of BAP 1.0 mg/l. The highest average value of shoot regeneration was observed 10–15 days after cultivation on DKW medium. A BAP concentration of 1.0 mg/l caused earlier induction of the main shoot without necrosis. Callus was observed on other variants of nutrient media (Fig. 4).

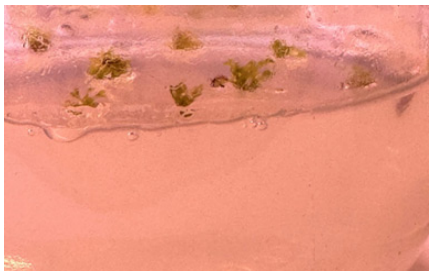
In the micropropagation of plants, the main goal is to obtain the maximum number of genetically identical shoots that can easily take root, acclimatize, and grow successfully under field conditions [34]. Among plant grow the regulators, cytokinins are the most commonly used. Cytokinins in plants regulate shoot branching, initiation of apical growth, and other critical growth processes [35].



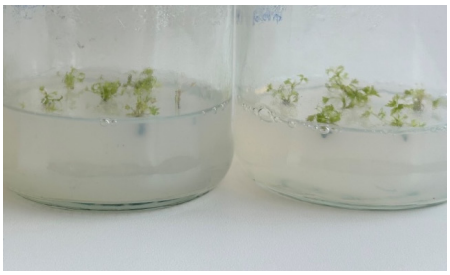
A) Explants on QL medium



B) Explants on DKW medium



C) shoots on the 21st day of cultivation on DKW medium



D) shoots on the 40th day of cultivation on DKW medium

Figure 4. Regel pear shoots formed on regeneration medium

The widely used synthetic cytokinin BAP (6-benzylaminopurine) is effective, versatile, and can be rapidly metabolized in plant tissues [36, 37]. BAP is often included in micropropagation protocols for various pear species [38, 39, 40, 41].

The optimal nutrient medium for the formation of the main shoot in Regel pear was found to be the DKW nutrient medium. This medium was used for the multiplication of Regel pear shoots. To select the optimal multiplication conditions, Regel pear shoots measuring about 0.9 cm were cultivated in DKW medium with various concentrations of plant growth regulators. The concentrations of growth regulators BAP, GA (gibberellic acid), and IBA (indole-3-butyric acid) had different effects on the multiplication of shoots (Table 2). The largest number of multiplied shoots perex plant (10.60±0.53) was observed on DKW medium with BAP 1.0 mg/l and GA 0.2 mg/l (Fig. 5). Shoot proliferation increased with increasing BAP concentration to 1.0 mg/l in treatments IV, VII, and X. The presence of IBA in the nutrient medium did not significantly differ from option X, where IBA was absent.

Micropropagation protocols have been developed for the main varieties of *P. communis* L. [42, 43, 44, 45, 46]. While some studies have been conducted on wild and domesticated *Pyrus* species, there is a lack of scientific research on the micropropagation of Regel pear. This study addresses that gap by providing a detailed protocol for the effective micropropagation of Regel's pear using optimal concentrations of plant growth regulators.

Table 2

Optimizing the multiplication of the Regel pear

Treatment	Plant growth regulators, mg/l			Phenological parameters		
	BAP	IBA	GA	Shoot height, cm	Shoots, pcs.	Number of leaves, pcs
I	-	-	-	1.25±0.04	1.07±0.27	5.60±0.12
II	0.1	0.1	0.2	0.96±0.04	2.03±0.35	13.83±0.11
III	0.5	0.1	0.2	1.07±0.08	4.37±0.56*	18.53±0.14*
IV	1.0	0.1	0.2	0.92±0.04	8.43±0.59*	35.73±0.15*
V	0.1	0.5	0.2	1.36±0.04	4.70±0.54*	23.30±0.15*
VI	0.5	0.5	0.2	1.42±0.05	7.90±0.45*	22.50±0.17*
VII	1.0	0.5	0.2	1.68±0.04*	9.77±0.69*	61.73±0.27*
VIII	0.1	-	0.2	0.57±0.05*	4.87±0.74*	19.37±0.25*
IX	0.5	-	0.2	1.50±0.06	5.77±0.55*	41.97±0.31*
X	1.0	-	0.2	0.71±0.06*	10.60±0.53*	65.40±0.29*

A) Shoots with BAP 1.0 mg/l, GA 0.2 mg/l, IBA 0.5 mg/l

B) Shoots with BAP 0.1 mg/l, GA 0.2 mg/l

C) Shoots with BAP 0.1 mg/l, GA 0.2 mg/l, IBA 0.1 mg/l

D) Shoots with BAP 0.1 mg/l, GA 0.2 mg/l, IBA 0.5 mg/l

Figure 5. Multiplicated shoots of Regel pear

Thus, the most optimal concentration of growth regulators for the formation of shoots that can be used for rooting *in vitro* of Regel pear is BAP 1.0 mg/l, IBA 0.5 mg/l and GA 0.2 mg/l.

Conclusion

Work has been carried out to establish scab-resistant Regel pears *in vitro* culture. The most optimal sterilization for Regel pear is the treatment of explants with 12 % H_2O_2 with an exposure time of 5 minutes, where the viability was 70 %. Conditions have been selected for the formation of the main shoot and multiplication of the Regel pear; the most optimal concentration of growth regulators for the formation of shoots of the Regel pear is BAP 1.0 mg/l, IBA 0.5 mg/l and GA 0.2 mg/l.

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Эндемикалық түр Регель алмұрты үшін *in vitro* өсіру жағдайларын оңтайландыру

Өсімдіктердің әртүрлілігінің азаюы климаттың өзгеруінен, өсімдіктердің аурулары мен адам әсерінен туындаған жаһандық мәселе. Бұл биоалуан түрлілікке теріс әсер етіп, өз кезегінде азық-түлік қауіпсіздігі мен экожүйенің тұрақтылығы мәселелеріне әкеледі. Жабайы өсімдік түрлерін сақтау

ерекше маңызға ие, себебі оларда биотикалық және абиотикалық факторларға төзімділікті қамтамасыз ететін гендер болуы мүмкін. Қазақстанда құрғақшылық пен ауруға төзімділік белгілері бар эндемикалық түр — Регель алмұрты өседі. Зерттеу барысында олардың өскен жерінен үлгілерді іздестіру және жинау жұмыстары жүргізілді. Регель алмұртының паршаға төзімді үлгілері SSR маркерінің көмегімен анықталды. Бұл экономикалық құнды түрді сақтау үшін алғаш рет *in vitro* өсіру шарттары оңтайландырылды. *In vitro*-ға дақылды енгізу және регенерациялау үшін стерилизация протоколы әзірленді. 1,0 мг/л, ИМҚ 0,5 мг/л, ГҚ 0,2 мг/л БАП-пен толықтырылған DKW қоректік ортасының мультипликация шарттары таңдалды. Бұл жұмыс генетикалық әртүрлілікті ұзақ мерзімді сақтауды қамтамасыз етуге және өзгермелі қоршаған орта жағдайларына ауылшаруашылығының тұрақтылығын арттыруға бағытталған.

Кілт сөздер: Pyrus regelii, *in vitro* дақылы, эндемик, микроклональды көбею.

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Оптимизация условий культивирования *in vitro* эндемичного вида груши Регеля

Сокращение растительного разнообразия является глобальной проблемой, вызванной изменением климата, болезнями растений и антропогенным воздействием. Это негативно сказывается на биоразнообразии, что, в свою очередь, приводит к проблемам продовольственной безопасности и устойчивости экосистем. Особую важность представляет сохранение диких видов растений, так как они могут содержать гены, обеспечивающие устойчивость к биотическим и абиотическим факторам. В Казахстане произрастает эндемичный вид — груша Регеля, который обладает признаками устойчивости к засухе и болезням. В ходе исследования проведены работы по поиску и сбору образцов в месте их произрастания. Определены устойчивые образцы груши Регеля к парше с использованием SSR-маркера. Впервые были оптимизированы условия культивирования *in vitro* для сохранения этого хозяйственно-ценного вида. Отработаны стерилизация для введения в культуре *in vitro* и регенерация. Подобраны условия мультипликации — питательная среда DKW с БАП 1,0 мг/л, ИМК 0,5 мг/л, ГК 0,2 мг/л. Эти усилия направлены на обеспечение долгосрочной сохранности генетического разнообразия и повышение устойчивости сельского хозяйства к изменяющимся условиям окружающей среды.

Ключевые слова: Pyrus regelii, *in vitro,* культура, эндемик, микроклональное размножение.

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