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ORIGINAL ARTICLE

Development of an in vitro conservation protocol for the endemic species *Pyrus regelii*

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Abstract

The preservation of genetic resources of wild relatives of cultivated plants, such as *Pyrus regelii*, is crucial for maintaining biodiversity, ensuring food security, and supporting the sustainable development of ecosystems. *P. regelii* is an endemic, relict species that is native to Kazakhstan and a very good research plant material due to its agronomically valuable traits, such as drought tolerance and disease resistance. Therefore, this study aimed to develop an effective in vitro conservation protocol for *Pyrus regelii* by investigating the effects of various plant growth regulators on its in vitro multiplication, in vitro rooting, and slow-growth storage phases. An effective micropropagation protocol was developed using the plant growth regulators meta-topolin (1.0 mgL^{-1}) and gibberellic acid (0.2 mgL^{-1}), where 29.47 microshoots per explant were obtained. For in vitro rooting, the most effective medium was ½ WPM, with an average of 3.1 roots formed per explant and rooting percentage of 77%. A slow-growth storage technique at +4 °C was also developed, in which the combination of abscisic acid (1.0 mgL^{-1}) and mannitol (2%) in ½ WPM medium effectively suppressed microshoot growth while maintaining plant viability for 4 month without re-cultivation. The results suggest that micropropagation and slow-growth storage at +4 °C can be used to conserve the endemic, relict *P. regelii*.

Key message

An effective in vitro protocol was developed for conserving *Pyrus regelii* through optimized micropropagation and slow-growth storage, ensuring long-term preservation of this drought- and disease-tolerant wild pear species.

Keywords *Pyrus regelii* · In vitro · Micropropagation · Slow-growth storage · In vitro rooting

Introduction

The biomass of all plants from pre-human times has reduced by 50% across millennia due to human activities (Bar-On et al. 2018), and 39% of vascular plants are threatened with extinction (Nic Lughadha, 2020). The relentless growth of human population on earth has had a devastating impact on plant biodiversity worldwide, and many of those with pharmacological properties, nutritional value, and ornamental appeal are now threatened with extinction (Almond, 2020; Balding and Williams, 2016). Hence, there is an urgent need to understand and conserve these plants because biodiversity in nature is the foundation of life on Earth.

Pyrus regelii is one of those species that has been negatively affected by human activity. The plant grows in the Western Tien Shan, Karatau, and Pamir-Altai regions in Kazakhstan, which are considered to have the

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highest population of endemic plant species. The southeastern regions of Kazakhstan are the northern border of this species. *P. regelii* is an important material for geobotanical studies on the formation of unique communities of mountain ecosystems in Central Asia (Abduhadyr et al. 2024; Bell and Itai 2010; Kubentayev et al. 2024; Teng 2017; Vincent et al. 2013).

P. regelii is a small tree that may grow up to 10 m. Its branches spread widely, and its thorns are long and thin. The buds are bare and acute, with widely separated, triangular, and pointed sepals. The leaves are completely bare, light green, shiny, thin-skinned, and up to 8–9 cm long. The flowers have a diameter of 2–2.5 cm, with the petals having a very short claw. The fruits are pear-shaped or sometimes flattened-spherical. *P. regelii* is found on dry rocky slopes, cliffs, and sometimes in valleys on very deep, moist soils at an altitude of 1000–2000 m above sea level. It is an extremely drought-resistant type of pear that is sometimes used as a rootstock for cultivation of pear trees, and it is also frost-resistant (Komarov 1939).

The genus *Pyrus* is believed to have originated during the Tertiary period, approximately 65–55 million years ago, in the mountainous regions of present-day southwestern China (Wu et al. 2018). From this center of origin, the lineage gradually dispersed along various mountain systems, expanding both eastward and westward. This wide geographic spread led to the divergence of the genus into two primary groups: Asian (oriental) and European (occidental) pears, each developing unique characteristics shaped by their respective environments. Phylogenetic and morphological evidence supports the monophyly of the genus *Pyrus* and its division into two major clades or subgenera: the western subgenus *Pyrus* and the eastern subgenus *Pashia*.

Within this taxonomic framework, *P. regelii* is part of the West Asian lineage and belongs to the subgenus *Pyrus*, which encompasses occidental species native to Europe, North Africa, and Western to Central Asia (Zheng et al. 2014). *P. regelii* is geographically situated in Central Asia, west of the distribution range of East Asian (oriental) pears, and is recognized as an early-diverging, phylogenetically distinct species within the West Asian group (Quinet and Wesel 2019).

Several phylogenetic studies have shown that *P. regelii* is one of the earliest offshoots in the genus *Pyrus* and has unique genetic characteristics. *P. regelii* has dissected adult leaves and a small number of locules in the flower that indicate similarity with the ancestral form. Chloroplast DNA analysis has shown that *P. regelii* is a relatively ancient offshoot within the genus *Pyrus* (Zheng 2014; Volk 2019; Teng 2018), and it can be assumed that the species is a relict. Therefore, this species plays a key role in understanding the early evolutionary history and divergence patterns of the genus *Pyrus*.

The areas occupied by *P. regelii* have gradually reduced in recent year due to anthropogenic activity, including economic activity and felling of trees. *P. regelii* is listed in the Red Book of the South Kazakhstan Region (Turdiev et al. 2023; Kurmantaeva et al. 2017). This species need to be preserved due to its phylogenetic significance and economically valuable traits. Moreover, *P. regelii* can be incorporated into breeding programs to develop new stress-resistant pear cultivars. The development of such resistant varieties will contributes to global food security (Chaudhry and Sidhu 2022).

One of the effective methods for conserving valuable plant species is the use of in vitro techniques. Currently, there are three main approaches to in vitro plant conservation: maintenance under active growth conditions, slow-growth storage at low temperatures (+ 2–15 °C), and cryopreservation in liquid nitrogen (− 196 °C) (Chokheli et al. 2020). These technologies enable the preservation and reintroduction of plants for biodiversity conservation and inclusion in breeding programs. Effective in vitro conservation protocols under active growth conditions such as micropropagation, have been developed for various plant species, including different pear species (Kaviani et al. 2023; Alexandri et al. 2023; Kirillov et al. 2024; Lotfi et al. 2020a). Additionally, protocols for slow-growth storage have been established for various pear species (Reed and DeNoma 2014; Chauhan et al. 2019). However, no studies on micropropagation and slow-growth storage of *P. regelii* have been conducted to date. Therefore, the present study aimed to develop an effective in vitro conservation protocol for this endemic and relict pear species by investigating the effects of various plant growth regulators on its in vitro multiplication, rooting, and slow-growth storage phases.

Materials and methods

Research objects

One-year-old shoots of the wild, endemic *P. regelii* were collected in the Sairam-Ugam National Park, Turkistan region, Kazakhstan and used as the research material. The microshoots obtained via in vitro culture were used for micropropagation, in vitro rooting, and slow-growth storage experiments.

Research methods

Collection and identification of plant material

Genomic DNA was extracted from the young leaves of maternal *P. regelii* using the cetyltrimethylammonium bromide (CTAB) method (Doyle 1991), with minor modifications in the extraction buffer. The DNA purity was checked on 1.5% agarose gel electrophoresis and using values of the 260/280 nm ultraviolet (UV) absorbance ratio. Polymerase chain reaction (PCR) was used for DNA amplification, and the set of primers used had the following sequences: rbcL (forward: 5'ATGTCACCACAAACAGAGACTAAAGC3') and rbcL (reverse: 5'GTAAAATCAAGTCCACCACG3') (Costion et al. 2011). The expected product size was 599 bp with an annealing temperature of 55 °C.

Amplification was performed in a 30-µl PCR reaction mixture comprising 1 × buffer, 0.2 µl of each dNTPs, 0.4 pmol Taq polymerase, 30 ng/µl template DNA, 0.5 µl of each primer, 1.5 µL $MgCl_2$, and 16.5 µl nuclease-free water. The PCR reaction steps were as follows: preheating for 4 min at 95 °C; 35 cycles each at 95 °C (40 s), 35 °C (55 s), and at 72 °C (1 min); and a final extension step at 72 °C (1 min) (Eppendorf MasterCyclerPro, Germany). After amplification, the PCR products were resolved in a 1.5% agarose gel (Himedia, Mumbai, India) and stained with ethidium bromide (0.4 µg/ml). DNA digested with 700 bp DNA Ladder (Fermentas) was used as the DNA marker, and bands were visualized under UV light and photographed using Gel Doc equipment (Bio Rad, USA).

The PCR reaction was performed at least twice for reproducibility of results. Purification of the PCR products was carried out using SAP (Shrimp Alkaline Phosphatase) and ExoI (ThermoScientific, USA) enzymes. The reactions were carried out in a 20-µl reaction mixture containing 1 × SAP buffer, 10 µl of PCR product, 3 units of exonuclease ExoI, and 1 unit of SAP and incubated at 37 °C for 30 min, followed by inactivation of the enzyme at 75 °C for 15 min. The initial incubation hydrolyzed the excess primers and dephosphorylated the nucleotides, and the second, high-temperature incubation inactivated the enzymes, which then minimized the loss of the PCR product allow possible further sequencing without further purification using the columns.

DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, USA), followed by analysis of the reaction products on an automated sequencer (Applied Biosystems 3730 DNA Analyzer, Tokyo, Japan). The nucleotide sequences of the analyzed samples were retrieved and edited using the SeqMan program (Allex et al. 1999).

Establishment of in vitro culture

For the establishment of in vitro culture of *P. regelii*, one-year-old axillary buds were used. The annual shoots were divided into segments, each containing a single axillary bud. Prior to sterilization, the explants were washed in a soapy solution on a magnetic stirrer for 3–4 h. Sterilization was carried out under laminar flow conditions using 12% H_2O_2, with an exposure time of 5 min. Before sterilization, the axillary buds were briefly treated with 70% ethanol to enhance detergent penetration (Dyussembekova et al. 2024).

Micropropagation

After obtaining the main shoot, medium experiments were conducted to optimize the hormonal composition of the woody plant medium (WPM) nutrient medium (McCownn and Sellmer 1987) for the micropropagation of *P. regelii* microshoots. The effects of different combinations of plant growth regulators (PGR), such as 6-benzylaminopurine (BAP; Sigma-Aldrich), meta-topolin (mT; Apollo Scientific), gibberellic acid (GA_3; Sigma-Aldrich), and indole-3-butyric acid (IBA; Sigma-Aldrich), were examined. The following concentrations of growth regulators were tested in the WPM medium: BAP and mT at 0.1 mgL^{-1}, 0.5 mgL^{-1}, and 1.0 mgL^{-1}; IBA at 0, 0.1 mgL^{-1}, and 0.5 mgL^{-1}; and GA_3 at 0.2 mgL^{-1}. Phenological measurements were recorded on day 1 and day 28 of cultivation. The explants were cultured in a growth chamber under a 16-hour photoperiod at 24–26 °C, each treatment consisted of 30 microshoots.

In vitro rooting

One of the key stages of micropropagation is the production of rooted microshoots for subsequent acclimatization to environmental conditions. To induce rooting in *P. regelii* microshoots, medium experiments were conducted to optimize the hormonal composition of the nutrient medium. For this purpose, WPM medium with half-strength macronutrients was used. The following treatments were tested: I– ½ WPM (control); II– ½ WPM with 0.1 mgL^{-1} IBA; III– ½ WPM with 0.2 mgL^{-1} IBA; IV– ½ WPM with 0.3 mgL^{-1} IBA. Phenological measurements were recorded on day 1 and day 40 of cultivation. The explants were cultured in a growth chamber under a 16-hour photoperiod at 24–26 °C, each treatment consisted of 30 microshoots.

Slow-growth storage (+ 4 °C) of *P. regelii* microshoots

To optimize the conditions for slow-growth storage of *P. regelii* microshoots, the effects of abscisic acid (ABA) and mannitol were investigated using WPM-based medium. A

total of 16 treatments were tested as follows: I– WPM (control); II– WPM with 1.0 mgL^{-1} ABA; III– WPM with 2.0 mgL^{-1} ABA; IV– WPM with 3.0 mgL^{-1} ABA; V– WPM with 2% mannitol; VI– WPM with 2% mannitol and 1.0 mgL^{-1} ABA; VII– WPM with 2% mannitol and 2.0 mgL^{-1} ABA; VIII– WPM with 2% mannitol and 3.0 mgL^{-1} ABA; IX– ½ WPM; X– ½ WPM with 1.0 mgL^{-1} ABA; XI– ½ WPM with 2.0 mgL^{-1} ABA; XII– ½ WPM with 3.0 mgL^{-1} ABA; XIII– ½ WPM with 2% mannitol; XIV– ½ WPM with 2% mannitol and 1.0 mgL^{-1} ABA; XV– ½ WPM with 2% mannitol and 2.0 mgL^{-1} ABA; and XVI– ½ WPM with 2% mannitol and 3.0 mgL^{-1} ABA. Microshoots were cultured at +4 °C under a 16/8-hour photoperiod in the experiment aimed at optimizing slow-growth storage conditions. The cultivation period lasted for 4 months, and each treatment consisted of 30 microshoots.

The pH for all culture media was adjusted to 5.8 using 0.5 mol/l NaOH prior to autoclaving at 121 °C for 20 min (MELAtronic 23, Berlin, Germany). All the in vitro plant cultures were maintained in a glass jar (500 ml with 50 ml medium), with five explants per jar. The cultures were maintained in a plant-growth facility at 24–26 °C with a 16-h photoperiod provided by fluorescent lamps (tubes, length 1.20 m) of two red lights (Osram L 36 W/77T8 Fluora) and two warm white lights (Osram L 36 W/830warm white).

Statistical analysis

The experimental results were analyzed using analysis of variance (ANOVA), and significant differences were determined using Tukey's post hoc test in SPSS 23.0 (IBM Inc., New York, USA). Data are presented as mean ± standard error. For analysis results at multiplication stage Duncan's multiple range test (DMRT) at a significance level of $P \leq 0.05$ was used to determine significant differences among treatment means. The results were presented as means ± standard errors (SE). All statistical analyses were conducted using the R software (version 4.5.0) with the "agricolae" package. Each treatment consisted of 30 microshoots, experiments were conducted in three replicates.

Results

Collection and identification of plant material

Plant materials of the endemic *P. regelii* were collected in the Sairam-Ugam State National Nature Park (Fig. 1; Table 1).

Species identification of the four collected *P. regelii* samples was carried out using the rbcL marker. The effectiveness of the rbcL marker for amplification and successful retrieval of nucleotide sequences for further analysis was confirmed. The minimum and maximum sequence lengths for the rbcL region ranged from 534 to 560 base pairs (bp). The guanine and cytosine (GC) content, which provides additional insights into the structural features of the studied *P. regelii* regions, ranged from 42.9 to 43.2%.

All the nucleotide sequences were manually edited using SeqMan software to eliminate potential errors and improve data accuracy. Sequence similarity was assessed using the BLAST method in the NCBI database to evaluate the degree of similarity with other known sequences. The sequencing results were submitted to the international NCBI database, where each of the four studied samples was assigned an accession number for the rbcL primer (Table 2).

Establishment of *Pyrus regelii* in vitro culture

For establishment of in vitro culture, one-year-old shoots were divided into segments containing a single axillary bud

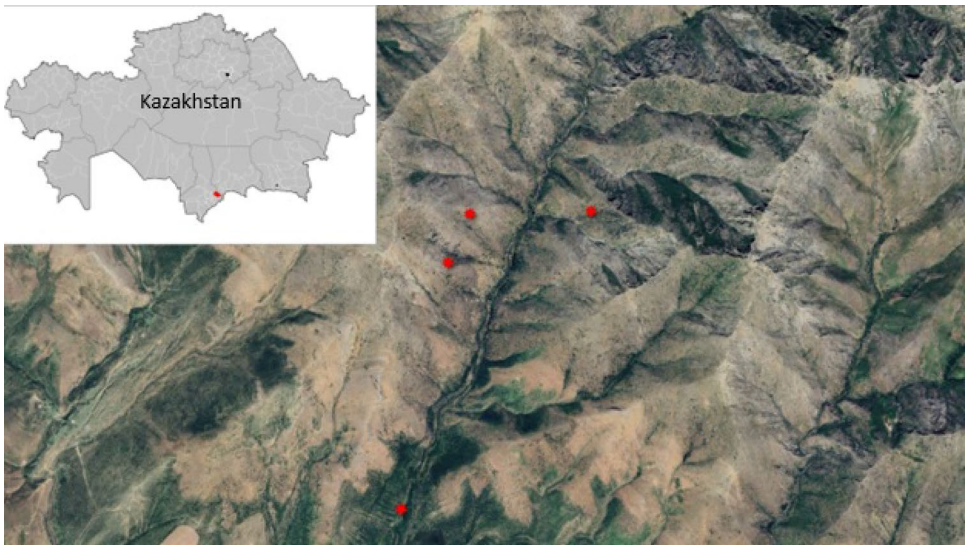

Fig. 1 Collection site of *P. regelii* in the Sairam-Ugam State National Nature Park, Kazkhstan Natural populations of *P. regelii* were identified, and shoots and leaves were collected from four trees (Fig. 2). *Explanation*: Red color in both maps means sampling site

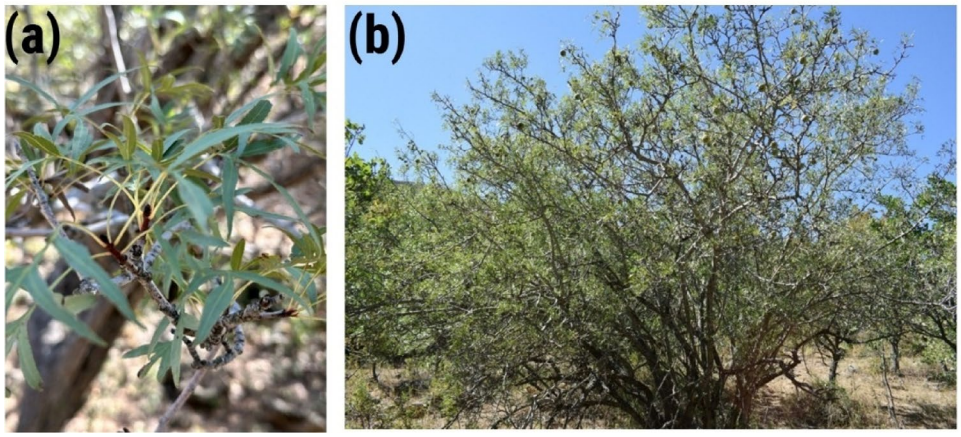

Fig. 2 *P. regelii* mature trees in its natural habitat (**a**) One-year shoots of *P. regelii*; (**b**) Mature tree of *P. regelii*

Table 1 Location and number of 4 trees *P. regelii* in natural habitat

No	Longitude	Latitude	Altitude	Collection place
1	E070°15.59'	N42°41.32'	930 m	Turkistan Region, Tulkubas District, Sairam-Ugam State National Park.
2	E070°15.48'	N42°41.14'	910 m	
3	E070°15.24'	N42°40.23'	820 m	
4	E070°16.20'	N42°41.33'	950 m	

Table 2 Accession numbers obtained in the NCBI database

Sample number	GC content, %	Accession number
1	42.9	PV110019
2	43.2	PV110020
3	43.2	PV110021
4	43.0	PV110022

(Fig. 3). In our previous study, the most effective method for sterilizing axillary buds for the establishment of in vitro culture was treatment with 12% H_2O_2 for 5 min (Dyussembekova et al. 2024).

After sterilization, apices were isolated from axillary buds under a binocular microscope (Olympus SZ51, Japan) for the establishment of in vitro culture (Fig. 4).

Selection of plant growth regulators for in vitro multiplication of *Pyrus regelii*

To evaluate the multiplication of *P. regelii* microshoots, the effects of various concentrations of PGRs, BAP and mT combined with GA_3 and IBA, were tested using WPM medium.

The results demonstrated that the selection of exogenous PGRs in in vitro culture played a crucial role (Table 3; Fig. 5). The data are presented as growth increments for all parameters. The statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at a significance level of $p \leq 0.05$. The results are presented as "mean value ± standard error (SE)". Mean values followed by the same letters are not significantly different ($p > 0.05$), while different letters indicate statistically significant differences between treatments ($p \leq 0.05$).

The results demonstrated that the selection of exogenous PGRs in in vitro culture played a crucial role (Table 3; Fig. 5). The data are presented as growth increments for all parameters. In this study on micropropagation of *P. regelii*, all the treatments supplemented with plant growth regulators significantly enhanced morphogenetic activity compared to

Fig. 3 Shoots of *P. regelii* for sterilization and establishment in vitro culture

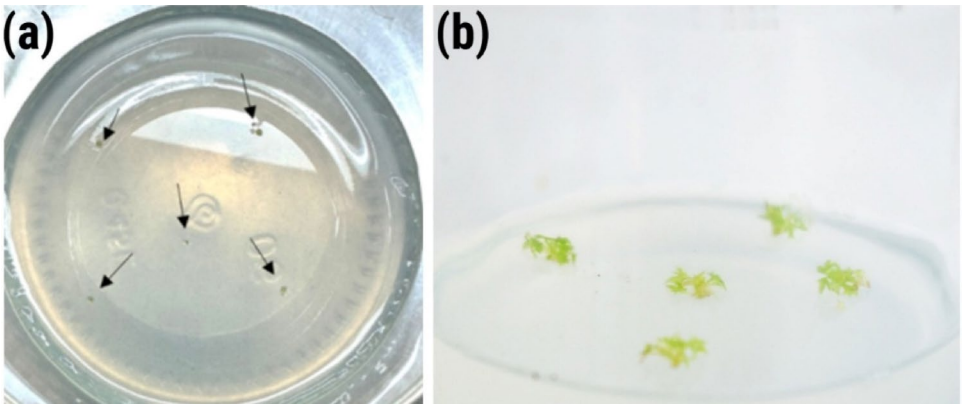

Fig. 4 Apices established into in vitro culture. (**a**) Explants on 1 st day of cultivation; (**b**) Explants on 21 st day of cultivation

Table 3 Results of the study on the effect of plant growth regulators during the multiplication stage of *Pyrus regelii*

Treatment (T)	Media	Plant growth regulators, mgL^{-1}				Phenological measurements of growth increments		
		BAP	mT	IBA	GA_3	Shoot height, cm	Number of microshoots, pcs	Number of leaves, pcs
TI	WPM	–	–	–	–	0.31 ± 0.07^{j}	1.53 ± 0.17^{m}	13.87 ± 0.38^{r}
TII		0.1	–	–	0.2	0.89 ± 0.06^{hi}	10.10 ± 0.21^{h}	67.83 ± 0.66^{h}
TIII		0.5				1.08 ± 0.07^{gh}	7.30 ± 0.24^{j}	52.37 ± 0.53^{l}
TIV		1.0				1.59 ± 0.09^{de}	11.93 ± 0.26^{g}	84.43 ± 0.56^{e}
TV		0.1		0.1		0.80 ± 0.06^{i}	5.67 ± 0.14^{k}	36.33 ± 0.47^{o}
TVI		0.5				0.81 ± 0.07^{i}	8.20 ± 0.21^{i}	54.80 ± 0.51^{k}
TVII		1.0				0.78 ± 0.06^{i}	2.83 ± 0.15^{l}	19.90 ± 0.47^{q}
TVIII		0.1		0.5		0.91 ± 0.05^{hi}	3.47 ± 0.17^{l}	26.93 ± 0.52^{p}
TIX		0.5				0.88 ± 0.05^{hi}	6.07 ± 0.23^{k}	39.03 ± 0.61^{n}
TX		1.0				0.74 ± 0.06^{i}	7.47 ± 0.17^{j}	56.27 ± 0.72^{k}
TXI		–	0.1	–	0.2	2.93 ± 0.06^{a}	19.77 ± 0.25^{d}	78.57 ± 1.04^{f}
TXII			0.5			2.95 ± 0.06^{a}	27.37 ± 0.34^{b}	111.63 ± 0.68^{b}
TXIII			1.0			2.51 ± 0.03^{c}	29.47 ± 0.25^{a}	118.20 ± 0.74^{a}
TXIV			0.1	0.1		1.25 ± 0.06^{gh}	11.80 ± 0.32^{g}	47.10 ± 0.52^{m}
TXV			0.5			1.02 ± 0.06^{fg}	17.87 ± 0.29^{e}	64.17 ± 0.42^{i}
TXVI			1.0			1.07 ± 0.07^{h}	20.20 ± 0.35^{d}	76.43 ± 0.41^{g}
TXVII			0.1	0.5		1.70 ± 0.06^{d}	15.50 ± 0.21^{f}	58.97 ± 0.62^{j}
TXVIII			0.5			1.41 ± 0.07^{ef}	22.13 ± 0.30^{c}	92.57 ± 0.81^{c}
TXIX			1.0			1.96 ± 0.06^{b}	21.93 ± 0.39^{c}	89.50 ± 0.65^{d}

Means followed by the same lowercase letter within a column are not significantly different according to Duncan's multiple range test (DMRT) at $p \leq 0.05$

the control (Treatment I). In particular, the media supplemented with growth regulators notably increased the number of shoots, their length, and the number of leaves.

In the experiment, the number of microshoots per explant ranged from 1.53 to 29.47. The highest number of microshoots was observed in treatment XIII, which contained 1.0 mgL^{-1} mT, 0.2 mgL^{-1} GA_3, and 0.5 mgL^{-1} IBA, and in treatment XII, with 0.5 mgL^{-1} mT and 0.2 mgL^{-1} GA_3, and they were 29.47 and 27.37 microshoots per explant, respectively (Fig. 5).

The control treatment (I), without the addition of plant growth regulators, showed the lowest effect on growth parameters: shoot height was 0.31 cm, with an average of 1.53 microshoots and 13.87 leaves per explant. The introduction of various plant growth regulators significantly altered the morphogenetic response of the shoots.

The addition of BAP at concentrations ranging from 0.1 to 1.0 mgL^{-1} in combination with 0.1 and 0.5 mgL^{-1} IBA and 0.2 mgL^{-1} GA_3 led to an increase in the number of microshoots from 2.83 to 11.93 and number of leaves from 19.90 to 56.27. However, shoot height did not differ significantly. The highest average number of microshoots (up to 11.93) and leaves (up to 84.43) was observed in the treatment with the combination of 1.0 mgL^{-1} BAP and 0.2 mgL^{-1} GA_3, without IBA For multiplication, IBA at 0.1 and 0.5 mgL^{-1} in combination with BAP and GA_3 inhibited the formation of microshoots in *P. regelii*.

In addition to BAP, the effect of the cytokinin metatopolin (mT) on the multiplication of microshoots was also investigated. The use of mT showed a pronounced stimulatory effect, particularly at concentrations of 0.5 mgL^{-1} and 1.0 mgL^{-1}. The highest growth was observed in treatment

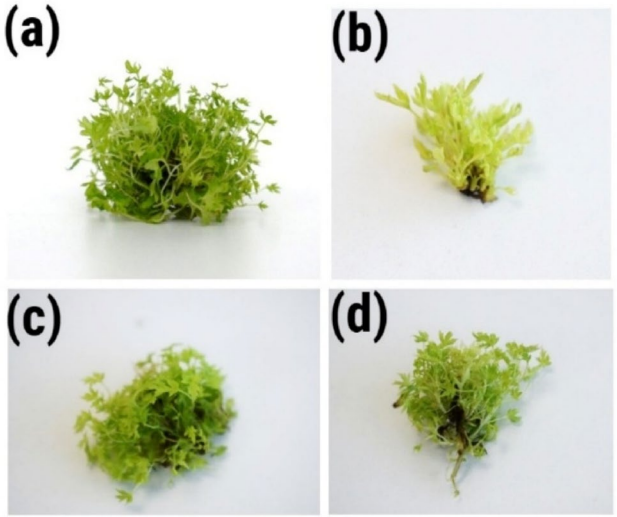

Fig. 5 Different multiplicated microshoots of *P. regelii* after experiment. (**a**) Microshoots on TXIII with 1.0 mgL^{-1} MT and 0.2 mgL^{-1} GA_3, average shoot number 29.47 per explant; (**b**) Microshoots on TX with 1.0 mgL^{-1} BAP, 0.5 mgL^{-1} IBA, and 0.2 mgL^{-1} GA_3, average shoot number 7.47 per explant; (**c**) Microshoots on TXVwith 0.5 mgL^{-1} MT, 0.2 mgL^{-1} GA_3, and 0.1 mgL^{-1} IBA, average shoot number 17.87 per explant; (**d**) Microshoots on TXIV with 0.1 mgL^{-1} MT, 0.2 mgL^{-1} GA_3, and 0.1 mgL^{-1} IBA, average shoot number 11.80 per explant

XIII, containing 1.0 mgL^{-1} mT and 0.2 mgL^{-1} GA_3, where the average shoot height reached 2.51 cm with 29.47 microshoots and 118.20 leaves per explant. The use of 0.5 mgL^{-1} mT in treatment XII also resulted in high values, with 27.37 microshoots and 111.63 leaves per explant.

Statistical analysis revealed significant differences among treatments in shoot height, number of shoots, and number of leaves per explant. With respect to the number of formed microshoots, the highest values were observed in TXIII (29.47 ±0.25) and TXII (27.37 ±0.34), which constituted a distinct and statistically significant group ('a' and 'b', respectively). In contrast, the control variant TI (1.53 ±0.17) showed the lowest performance and was significantly inferior to all other treatments, being assigned to group 'm'. Thus, meta-topolin at a concentration of 1.0 mgL^{-1} combined with 0.2 mgL^{-1} GA_3 was identified as the most effective cytokinin for micropropagation of *P. regelii* microshoots. Compared to BAP, this treatment resulted in the highest number of morphologically well-developed shoots.

Selection of plant growth regulators for in vitro rooting of *P. regelii*

In vitro rooting is the process of developing a root system in plants grown on nutrient media under sterile conditions. Successful rooting is a critical stage, as the formation of a well-developed root system determines the viability of the plant after transfer to soil conditions. Experiment was conducted to induce rooting in *P. regelii* under in vitro conditions. The WPM medium with half-strength macronutrients was used in combination with the auxin IBA at various concentrations. The results showed that root formation occurred by day 45 of cultivation (Fig. 6). Rooting was observed in the treatment without growth regulators (½ WPM) and in the treatment with 0.1 mgL^{-1} IBA. In the control treatment (½ WPM without growth regulators), the average shoot height was 2.31 cm, the number of roots was 3.1, and root length was 2.57 cm. No root formation was observed in two of the treatments (Table 4; Fig. 6).

Thus, among the tested concentrations of IBA, the highest frequency of root induction in *P. regelii* was observed on ½ WPM medium without the addition of growth regulators (Fig. 6). In this treatment, the rooting percentage of microshoots reached 77%.

Selection of growth inhibitors for slow-growth storage (+ 4 °C) of *P. regelii*

In our study, the effects of the osmotic agent mannitol and ABA on the slow-growth storage of *P. regelii* were examined using in vitro culture. These substances were used to

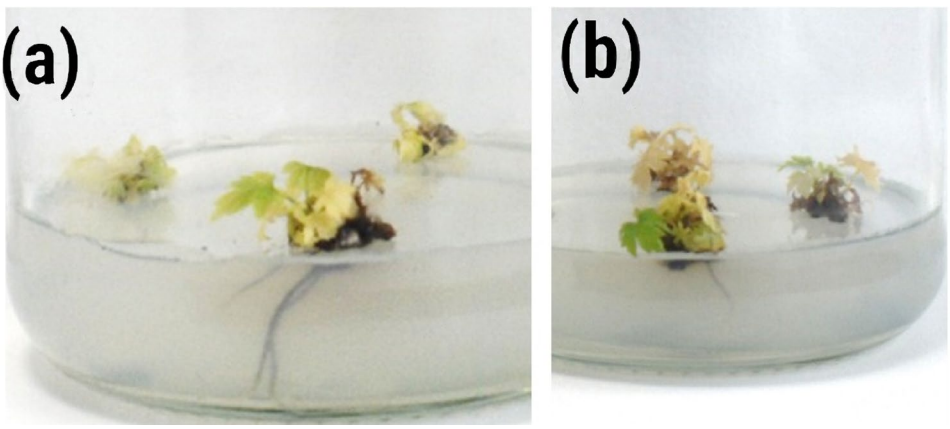

Fig. 6 Rooted on ½ WPM microshoots of *P. regelii*

Table 4 Results of the study on the effect of different concentrations of IBA on the in vitro rooting stage of *Pyrus regelii*

Treatment	Media	IBA, mgL^{-1}	Phenological measurements			
			Shoot height, cm	Number of roots, pcs	Root length, cm	Rooting percentage, %
I	½WPM	–	2.31 ± 0.09	3.10 ± 0.30	2.57 ± 0.36	77
II		0.1	1.81 ± 0.11	1.82 ± 0.11	1.56 ± 0.21	52
III		0.3	0	0	0	0
IV		0.5	0	0	0	0

Table 5 Results of the study on the effect of different growth inhibitors on the slow-growth storage of *Pyrus regelii* microshoots at + 4 °C

Treatment	Media	Growth inhibitors		1 st day of cultivation		120th day of cultivation		Accretion		Viability, %
		Mannitol, %	ABA, mgL^{-1}	Shoot height, cm	Number of leaves, pcs	Shoot height, cm	Number of leaves, pcs	Shoot height, cm	Number of leaves, pcs	
I	WPM	–	–	0.72 ± 0.03	4.80 ± 0.29	1.72 ± 0.04	10.87 ± 0.42	0.99 ± 0.03*	6.07 ± 0.32	100
II		–	1.0	0.74 ± 0.03	4.83 ± 0.30	1.14 ± 0.04	8.63 ± 0.53	0.40 ± 0.02*	3.80 ± 0.49	100
III		–	2.0	0.81 ± 0.02	4.40 ± 0.29	0	0	0	0	0
IV		–	3.0	0.77 ± 0.03	4.17 ± 0.30	0	0.00	0	0	0
V		2	–	0.74 ± 0.04	4.77 ± 0.28	1.32 ± 0.06	5.87 ± 0.39	0.58 ± 0.04*	1.10 ± 0.53	100
VI			1.0	0.78 ± 0.03	4.40 ± 0.34	0	0.00	0	0	0
VII			2.0	0.80 ± 0.03	4.43 ± 0.33	0.2	1.37 ± 0.41	−0.60 ± 0.07*	−3.07 ± 0.42	33
VIII			3.0	0.79 ± 0.03	4.50 ± 0.33	0	0.00	–	–	0
IX	½ WPM	–	–	0.76 ± 0.03	4.20 ± 0.27	0.64 ± 0.07	7.03 ± 0.37	0.34 ± 0.02*	2.83 ± 0.32	100
X		–	1.0	0.71 ± 0.03	4.83 ± 0.32	0.1 ± 0.25	4.93 ± 0.57	−0.07 ± 0.07*	0.10 ± 0.45	100
XI		–	2.0	0.78 ± 0.03	4.43 ± 0.32	0	0	0	0	0
XII		–	3.0	0.71 ± 0.03	4.30 ± 0.35	0	0	0	0	0
XIII		2	–	0.75 ± 0.03	4.70 ± 0.30	1.01 ± 0.04	5.53 ± 0.35	0.26 ± 0.02*	0.83 ± 0.17	100
XIV			1.0	0.76 ± 0.03	4.13 ± 0.31	0.81 ± 0.06	4.33 ± 0.38	0.05 ± 0.05*	0.20 ± 0.24	100
XV			2.0	0.72 ± 0.03	4.67 ± 0.34	0.09 ± 0.04	0.670.28	−0.64 ± 0.05*	−4.00 ± 0.4	17
XVI			3.0	0.73 ± 0.03	4.77 ± 0.31	0	0	0	0	0

*P < 0.05 (t-test). Less significant model terms are not shown

suppress active plant growth while maintaining viability for future use (Table 5; Fig. 7).

To optimize the conditions for slow-growth storage of *P. regelii* microshoots, the influence of 1.0 mgL^{-1}, 2.0 mgL^{-1}, and 3.0 mgL^{-1} ABA, with or without the addition of 2% mannitol, was investigated using WPM and ½ WPM media (Table 5; Fig. 7). Plants under the standard WPM medium without growth inhibitors (control) exhibited normal growth. After four months, the shoot height increased by 0.99 cm and the number of leaves reached 6.07 per explant, indicating that without growth inhibitors, plants continue active development, which is undesirable for slow-growth storage.

The addition of 1.0 mgL^{-1} ABA led to a significant reduction in growth: shoot height decreased to 0.40 cm and the number of leaves dropped to 3.80. This suggests that ABA effectively suppresses shoot and leaf development while maintaining 100% plant viability. However, the increase in the ABA concentration to 2.0 and 3.0 mgL^{-1} resulted in the death of the microshoots. In contrast, the combination of 2% mannitol with 1.0 mgL^{-1} ABA showed negative growth values (− 0.78 cm for shoot height and −4.40 for leaf number), indicating strong suppression of development and likely loss of viability. Negative values were recorded for growth increments, calculated as the difference between the final and initial measurements. Shoot height and the number of green leaves were used as indicators of growth, while microshoots exhibiting necrosis were excluded from the analysis.

The increase in ABA concentration to 2.0 mgL^{-1} in combination with mannitol also led to negative growth indicators and reduced microshoot viability to 33%, suggesting the death of most plants.

Weak growth was observed in the plants on ½ WPM medium without inhibitors: shoot height was 0.34 cm and the number of leaves was 2.83, but the microshoots remained viable. The addition of 1.0 mgL^{-1} ABA almost completely suppressed growth (− 0.07 cm in height and 0.10 leaves), but the number of microshoots remained unchanged, suggesting retained viability with nearly complete growth inhibition.

When 2% mannitol was added to the ½ WPM medium, shoot height decreased to 0.26 cm and leaf number to 0.83, with all microshoots remaining viable. However, the combination of 1.0 mgL^{-1} ABA and 2% mannitol in the ½ WPM

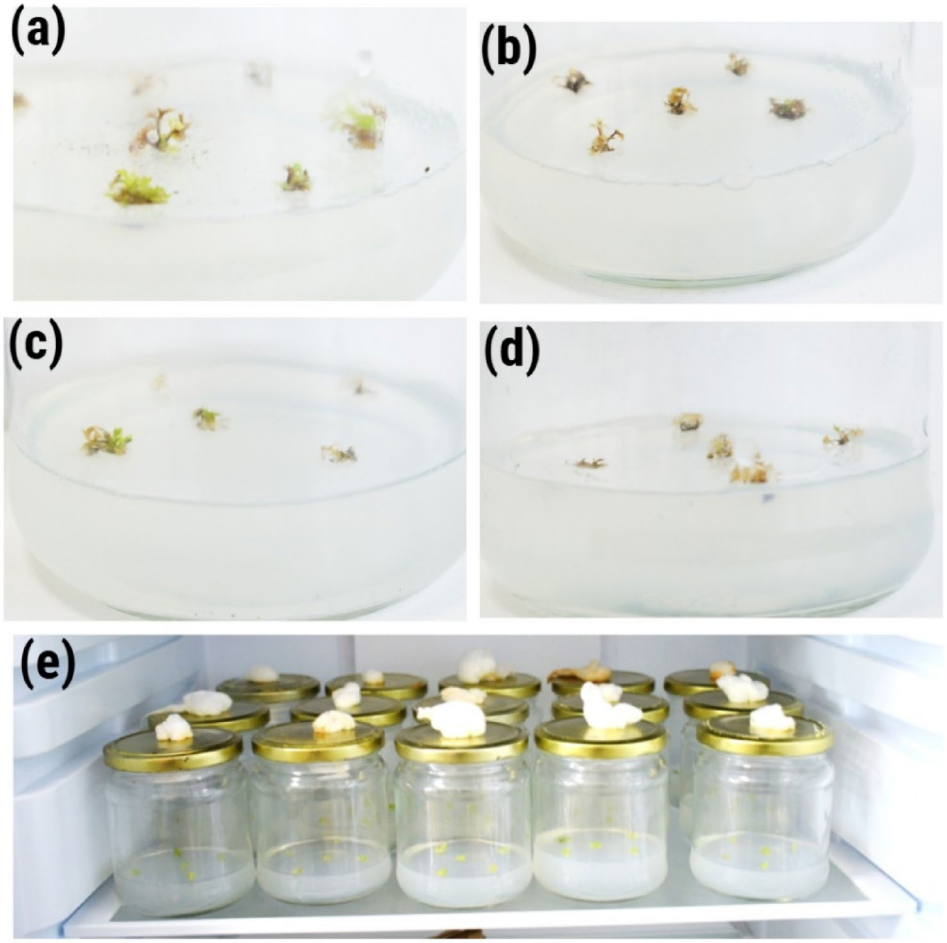

Fig. 7 Optimization of slow-growth storage conditions (+ 4 °C) for *P. regelii*. (**a**) Treatment XIV - ½ WPM with 2% mannitol and 1.0 mgL^{-1} ABA; (**b**) Treatment XII - ½ WPM with 3.0 mgL^{-1} ABA; (**c**) Treatment XV - ½ WPM with 2% mannitol and 2.0 mgL^{-1} ABA; (**d**) Treatment VIII WPM with 2% mannitol and 3.0 mgL^{-1} ABA; (**e**) Cultivation of microshoots at + 4 °C

Fig. 8 Recovered microshoots treated on ½ WPM with 2% mannitol and 1.0 mgL^{-1} ABA after slow-growth storage

medium resulted in almost no observable shoot growth after 4 months, but the viability remained at 100%, indicating that this treatment was effective for slow-growth storage.

For the slow-growth storage of *P. regelii* in vitro, the addition of abscisic acid and mannitol to the ½ WPM medium effectively suppressed plant growth while maintaining viability over a 4-month period. The optimal concentrations that inhibited microshoot growth were 1.0 mgL^{-1} ABA and 2% mannitol in ½ WPM medium, which minimized shoot and leaf development while preserving viable microshoots. For recovery after slow-growth storage, the microshoots were cultured on WPM medium supplemented with 1.0 mgL^{-1} mT and 0.2 mgL^{-1} GA_3. The recovery rate of *P. regelii* microshoots was 100% (Fig. 8).

Discussion

Micropropagation of woody plants is a vital technique in modern biotechnological research that enables the rapid and large-scale production of genetically identical clones of valuable tree species. This in vitro propagation method supports breeding programs and conservation of species with economically important traits. The regenerative capacity of plants depends on various factors, including the use of plant growth regulators, the composition of the basal medium, and the type of explant. It is important to note that plant tissue culture demonstrates strong species-specific and genotype-dependent responses (Long et al. 2022). One of the critical steps of micropropagation is the establishment of an effective sterilization protocol for in vitro culture initiation. Contamination at this stage can hinder the development of successful micropropagation protocols. Contaminants may include a wide range of microorganisms, such as bacteria, fungi, molds, and yeasts (Joint FAOIAEA, Division of Nuclear Techniques in Food and Agriculture 2004). Various surface sterilization techniques, involving chemical agents such as antiseptics, liquid detergents, mercuric chloride, or sodium hypochlorite, are used to prevent contamination (Seliem et al. 2020; Kushnarenko et al. 2022).

Currently, hydrogen peroxide is considered one of the safest and most effective sterilizing agents. For example, 10% $H_2O_2$for 10 min was effective for *Prunus persica* (Al-Ghasheem et al. 2018), and 5.5% H_2O_2 for 20 min was effective for *Staurogyne repens* (Köse et al. 2020). Hydrogen peroxide is widely used as a sterilizing agent for fruit crops in concentrations ranging from 3 to 30% (Ryago 2023). In the present study, the optimal sterilization condition for *P. regelii* was found to be 12% hydrogen peroxide for 5 min, which proved to be the most effective treatment for successful regeneration of this species (Fig. 4).

Micropropagation protocols have been developed for numerous plant species, including various *P.* species. Studies on *P. elaeagrifolia* have demonstrated the effectiveness of using benzyladenine (BA) for shoot proliferation and IBA for root induction both in vitro and ex vitro (Aygun and Dumanoglu 2015). BAP at a concentration of 1.0 mgL^{-1} was also successfully used in modified MS medium for *P. communis* and *P. syriaca* (Lotfi et al. 2020a). A combination of 5 µM BA with 0.0246 µM IBA yielded the highest microshoot proliferation in a study on *P. spinosa* (Tsoulpha et al. 2018). These findings highlight the need for cytokinin stimulation to achieve effective multiplication of *P. regelii* (Abdalla and Dobránszki 2024; Lotfi et al. 2020b).

In our previous study (Dyussembekova et al. 2024), we investigated the effect of plant growth regulators BAP, GA_3, and IBA in DKW medium (Driver and Kuniyuki 1984) during the multiplication stage of *P. regelii*. The results showed that the highest number of shoots 10.10 microshoots per explant was obtained with 1.0 mgL^{-1} BAP and 0.2 mgL^{-1} GA_3. However, the microshoots exhibited symptoms of chlorosis and leaf yellowing. We assumed this was due to the composition of the culture medium and decided to conduct a new experiment using a different medium, WPM, and included the cytokinin mT in the study.

DKW medium was originally developed for walnut (*Juglans*), whereas WPM is designed for hard-to-cultivate woody species. The total nitrate nitrogen content in DKW is approximately 4 to 5 times higher than in WPM due to the presence of NH_4NO_3 and $Ca(NO_3)_2{\cdot}4\ H_2O$. Additionally, DKW contains about twice the concentration of potassium (K^+) and calcium (Ca^{2+}) ions compared to WPM.

One of the main causes of leaf chlorosis in in vitro culture is an imbalance between nitrogen sources and iron availability in the medium. Excess nitrate can induce latent iron deficiency, leading to plant chlorosis. Iron chlorosis manifests as pale or yellowing young leaves (interveinal chlorosis) due to insufficient available iron, which is essential for chlorophyll synthesis (Chen et al. 2018; Liu et al. 2022). Therefore, we selected WPM medium to optimize the multiplication of the woody species Pyrus regelii, and the results are presented here for the first time.

In our study, the application of different plant growth regulators enabled the successful establishment of in vitro culture of *P. regelii* and revealed the significant role of cytokinin type on micropropagation efficiency. Media containing BAP promoted the formation of a large number of shoots, but the shoot quality was lower, characterized by thickened stems and small leaves, and showed signs of hyperhydricity. In contrast, the use of the aromatic cytokinin meta-topolin (mT) led to the development of physiologically healthier shoots with normal morphology. In all the treatments with mT, shoots were more vigorous, with significantly greater height, number of microshoots, and number of leaves compared to BAP. Additionally, no signs of vitrification or necrosis were observed in mT-treated shoots compared to those cultured on BAP-containing media. For example, the use of 1.0 mgL^{-1} mT in combination with 0.2 mgL^{-1} GA_3 resulted in an average of 29.47 microshoots per explant, whereas the same concentrations of BAP and GA_3 produced 11.93 microshoots per explant (Table 3). This difference is likely attributed to the mode of action and metabolism of meta-topolin, a naturally occurring aromatic cytokinin analog with a hydroxyl group, which may be metabolized differently in plants compared to BAP (Ahmad 2021). These findings align with those of previous studies on *Pyrus* species. For instance, Lotfi et al. (2020a) demonstrated that the replacement of BAP with aromatic topolin derivatives during micropropagation of *P. communis* eliminated shoot hyperhydricity and significantly improved shoot quality. In

their experiments, the use of 5 µM meta-meth-topolin riboside (MemTR) or meta-topolin riboside (mTR), instead of BAP, completely prevented the formation of vitrified shoots and increased the number of shoots with well-developed leaves (Lotfi et al. 2020b).

Other studies also confirm the advantages of aromatic cytokinins. Research on the pear rootstock 'OHF 333' (*P. communis*) showed that meta-topolin derivatives resulted in the longest shoots and the highest number of leaves compared to the control without cytokinins. Notably, at 6 µM mTR, the highest average shoot length and maximum leaf count per shoot were recorded. Although mT was not directly compared with BAP in that study, the efficacy of topolins was evident: without cytokinins, shoot multiplication was ineffective, whereas mT supplementation (6–9 µM) resulted in high proliferation rates and improved physiological condition of shoots, including low phenolic accumulation and high gas exchange activity (Dimitrova et al. 2024).

In a recent study on the wild pear *P. spinosa*, increased BAP concentration up to 20 µM significantly increased the multiplication rate (up to ~27 shoots per explant) but also introduced a risk of morphological abnormalities such as vitrification (Alexandri et al. 2023). Thus, meta-topolin not only ensures a high multiplication rate but also promotes the formation of physiologically healthy shoots that are suitable for rooting and successful acclimatization.

Many plants are capable of spontaneous root formation under in vitro conditions, but for most cultivated species, the addition of plant growth regulators such as auxins is required to stimulate rooting. Rooting process is particularly critical in micropropagation, as microshoots grown in vitro often have poorly developed or completely absent root systems, which reduces their chances of successful acclimatization. Auxins are key regulators that determine the success of in vitro rooting in fruit crops. Without the addition of auxins, many woody species exhibit poor adventitious root formation; for example, in pear microshoots, rooting on hormone-free media typically does not exceed 0–13% even after 60 days of cultivation. To promote rhizogenesis, IBA and naphthaleneacetic acid (NAA) are commonly used, but indole-3-acetic acid (IAA) is used less frequently. According to recent reviews, NAA is often the most effective auxin for in vitro rooting of pear, followed by IBA and IAA (Song et al. 2024).

A general principle is that without exogenous auxin, the basal parts of microshoots may fail to initiate root meristems, but the application of an optimal auxin concentration significantly increases rooting frequency and the number of roots. For instance, in apple, the use of IBA or NAA in rooting media significantly improves root formation (Sun et al. 2016). However, excessively high auxin concentrations can be detrimental, leading to excessive callus formation at the shoot base instead of normal root development (Edelmann 2022). Therefore, it is essential to select the appropriate type and concentration of auxin for each genotype to ensure optimal rooting efficiency without undesirable side effects. In a study on the cultivar 'Pyrodwarf', the highest number of roots was obtained using IAA at a concentration of 1 $mg \cdot L^{-1}$ (Kaviani et al. 2023).

In our study, the rooting percentage of *P. regelii* microshoots in the control treatment was significantly higher than that typically reported for other pear species. This finding indicates a strong inherent rooting ability in this species. It is possible that *P. regelii* possesses elevated levels of endogenous auxins or reduced levels of endogenous cytokinins, which facilitates root initiation without the need for exogenous hormonal stimulation. Additionally, the use of a nutrient medium with half-strength macronutrients (½ WPM) may itself have promoted rhizogenesis (Table 4; Fig. 6).

One of the major objectives for maintaining micropropagated cultures in in vitro collections is to slow down the growth of explants without compromising their viability. The so-called slow-growth storage approach allows the interval between subcultures onto fresh medium to be extended to several months or even years, thereby reducing labor input and minimizing the risk of somaclonal variation. The main strategies for growth retardation include lowering the cultivation temperature (commonly to +4 °C for cold-tolerant species), reducing light intensity or maintaining cultures in complete darkness, and modifying the composition of the nutrient medium (Trejgell et al. 2015; Rajasekharan and Sahijram 2015a, b; Sedlak et al. 2018).

In our experiment, a combined strategy was applied for the slow-growth storage of *P. regelii* shoots: low-temperature conditions at +4 °C, cultivation on ½ WPM medium supplemented with 2% mannitol, and 1.0 mgL^{-1} abscisic acid (Table 5, Figs. 7, 8). The selection of these components aligns with literature reports on the most effective methods for growth suppression. Mannitol, a non-reducing sugar alcohol, acts as an osmotic agent. At a concentration of approximately 2%, it increases the osmotic pressure of the medium by reducing the water uptake, which then limits cell expansion and meristematic division.

Modern protocols increasingly utilize mannitol in concentrations ranging from 1.5 to 3% in combination with sucrose or glucose to prolong the storage of cultures without causing irreversible metabolic disruptions (Linjikao et al. 2024; Chappell et al. 2020; Ahmed and Anjum 2010). For example, in apple, it has been shown that a medium containing 2% sucrose and 2% mannitol (without plant growth regulators) at +4 °C resulted in successful storage of microshoots for more than 36 months without subculturing (Benelli et al. 2022). Abscisic acid is another component

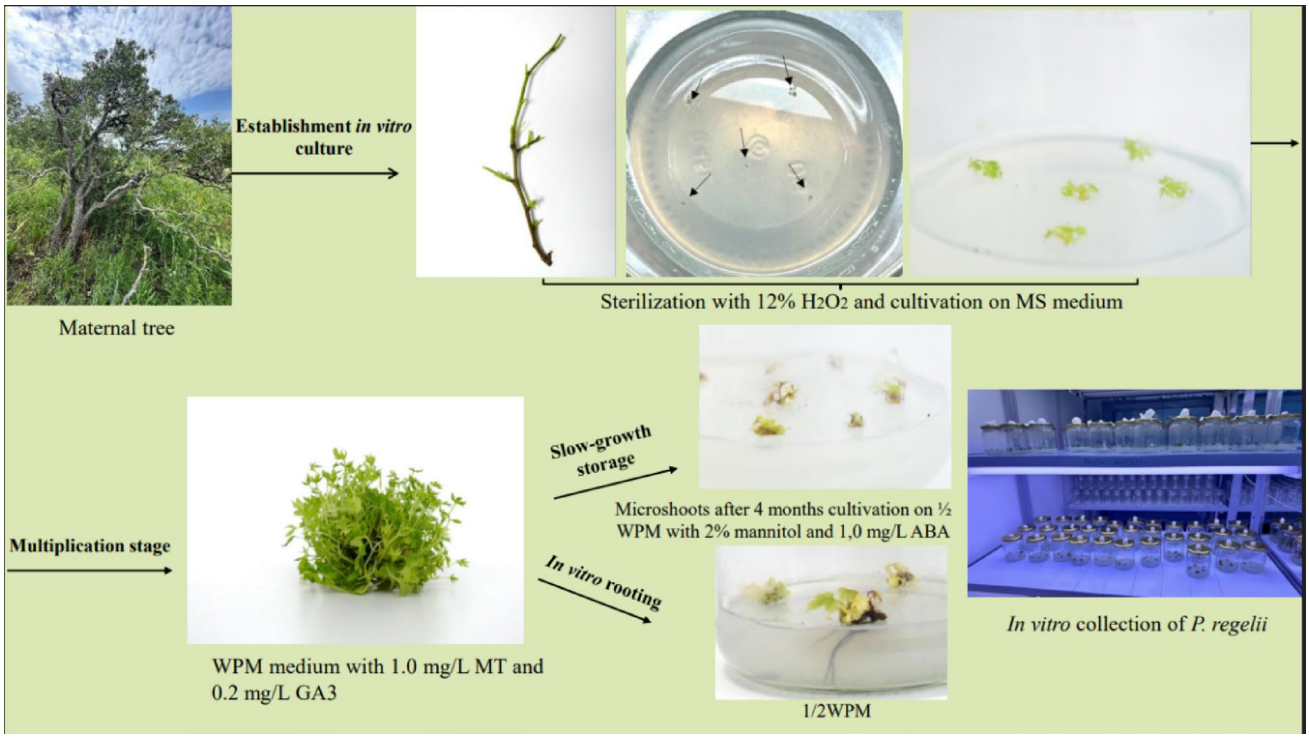

Fig. 9 Developed in vitro conservation protocol for *Pyrus regelii*

commonly added to storage media. This plant hormone plays a key role in inducing dormancy and inhibiting cell division. ABA suppresses the expression of genes involved in mitosis and cell expansion and promotes the synthesis of protective proteins and osmoprotectants, making it particularly useful for storing tissues at low temperatures (Pan et al. 2014; Mona et al. 2016).

There are several documented cases of successful ABA application in the storage of fruit crops. Studies on lilies and chestnut have also shown improved storage outcomes with the inclusion of ABA in the medium at low positive temperatures (Yun-peng et al. 2012; Capuana and Di Lonardo 2013). The presence of ABA is beneficial under cold conditions: that is, ABA enhances tissue tolerance to dehydration and cold stress to increase explant survival during long-term storage (Kovalchuk et al. 2009). This hormone effectively induces a physiological state that is analogous to winter dormancy. That is, it reduces respiration and cell division rates while stimulating the synthesis of stress-protective molecules, such as those that stabilize membranes and macromolecules.

The application of 1.0 mgL^{-1} ABA in combination with 2% mannitol in our experiment created optimal conditions for the slow-growth storage of *P. regelii* shoots (Table 5). The low positive temperature (+ 4 °C) significantly suppressed metabolic activity, whereas ABA enhanced this effect by preventing unwanted shoot development. Mannitol further reduced the water status of the tissues to potentially enhance the action of ABA, because mild dehydration can improve the ability of ABA to maintain viability by simulating a drought-tolerant physiological state (Trejgell et al. 2015).

These results are consistent with findings from other studies on in vitro storage of pear and related species. For example, the shoots of *P. communis* have been successfully stored at +4 °C without subculturing for up to seven months (Reed et al. 2014). Thus, we developed an in vitro conservation protocol for the endemic species *P. regelii*, as a contribution to biodiversity preservation (Fig. 9).

Conclusion

An in vitro propagation and slow-growth storage protocol was developed for the endemic and relict species *Pyrus regelii*. In vitro cul ture was established using 12% hydrogen peroxide for 5 min for sterilization, and apices were cultured on MS medium. Optimal conditions for microshoot multiplication were achieved with WPM medium supplemented with 1.0 mgL^{-1} meta-topolin and 0.2 mgL^{-1} GA_3. For slow-growth storage, cultivation on ½ WPM medium with 2% mannitol and 1.0 mgL^{-1} ABA proved effective, as it allowed a storage period of up to 4 months without subculturing. For in vitro rooting, ½ WPM medium was found to be the most suitable. Thus, an in vitro collection of *P. regelii* has been established for biodiversity conservation,

based on the developed protocol, which comprised micropropagation and slow-growth storage at +4 °C.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Damira Dyussembekova, Aidana Nurtaza, Galymzhan Zhenisbekuly and Gulmira Magzumova. The first draft of the manuscript was written by Damira Dyussembekova, Almagul Kakimzhanova and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Damira Dyussembekova: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Writing– original draft, Writing– review & editing. Aidana Nurtaza: Methodology, Investigation, Formal analysis. Gulmira Magzumova: Methodology, Investigation. Galymzhan Zhenisbekuly: Methodology. Almagul Kakimzhanova: Supervision, Project administration, Writing– review & editing.

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Data availability Data will be made available on request.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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