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Prospects for the conservation of *Fraxinus sogdiana* through micropropagation and slow growth storage approaches

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Abstract. *Rakhimzhanova A, Kali B, Zhumabay N, Tussipkan D, Manabayeva S. 2026. Prospects for the conservation of* Fraxinus sogdiana *through micropropagation and slow growth storage approaches. Biodiversitas 27 (4): d270421. https://doi.org/10.13057/biodiv/d270421.* *Fraxinus sogdiana* is a rare species listed in the Red Book of Kazakhstan, highlighting the urgent need for effective conservation strategies. This study aims to develop an effective micropropagation protocol, slow growth storage approaches and analyze phylogenetic relationships by comparing *F. sogdiana* from Kazakhstan with species from the NCBI database. The results of this study demonstrate that the DKW medium outperformed the MS medium in promoting shoot development. A DKW-based MP-V medium supplemented with 1.0 mg/L BAP, 0.1 mg/L NAA, and 0.5 mg/L gibberellic acid was found to be optimal for shoot proliferation. This resulted in maximum shoot lengths of 11.54 cm and an average of seven shoots per explant. High concentrations of BAP alone inhibited shoot formation. In slow-growth storage experiments, the addition of 0.5 mg/L CCC provided the best balance between shoot survival and growth retardation. In contrast, mannitol and ABA suppressed shoot development and decreased the survival rate. Based on their *ITS* patterns, five main groups were identified according to the sections of genus *Fraxinus*, including *Fraxinus*, *Sciadanthus*, *Melioides*, *Pauciflorae*, and *Ornus*. Notably, seven sequences from the species *Fraxinus angustifolia*, *Fraxinus obliqua*, *Fraxinus sogdiana*, and *Fraxinus turkestanica*, previously considered synonyms, were grouped together with strong bootstrap support. The results of the matK and rbcL gene patterns did not correspond to clearly defined taxonomic groups according to sections. The main finding of the matK gene region analysis was that the populations from Kazakhstan and China were closely related, as indicated by a high bootstrap value. These findings provide an effective protocol for preserving the genetic resources of *F. sogdiana*, as well as a phylogenetic framework to support future biotechnological and molecular studies.

Keywords: DNA barcoding, *Fraxinus sogdiana*, micropropagation, phylogenetic analysis, slow growth storage

INTRODUCTION

The genus *Fraxinus* comprises the two subgenera *Fraxinus* and *Ornus* and includes more than 50 species. *Fraxinus* L. is considered to have originated mainly in North America and also in eastern and western Asia and Europe (Masi et al. 2022; Meger et al. 2024; Richins et al. 2024). *Fraxinus sogdiana* Bunge is a relict species native to Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, China, and Pakistan. In Kazakhstan, it occurs in the Charyn Ash Grove of the Charyn State National Nature Park, where the grove covers about 5,000 ha, although ash stands occupy slightly more than 800 ha (Salmurzauly et al. 2024). Some trees in the Charyn Ash Grove are more than 300 years old.

Fraxinus sogdiana is categorized as “Least Concern” on the IUCN Red List, but because of declining natural populations, it is included in the Red Book of the Republic of Kazakhstan. Salmurzauly et al. (2024) and Sadyrova et al. (2025) assessed the ecological status of *F. sogdiana* in Charyn Canyon and reported major anthropogenic pressures, including deforestation, uncontrolled grazing, and accelerating habitat degradation. However, no specific conservation programs are in place, and active measures such as assisted regeneration, seed banking, and ex situ cultivation remain limited.

Several studies have explored regeneration pathways in *Fraxinus*, including shoot organogenesis (Tonon et al. 2001; Lee and Pijut 2017), epicotyl-derived micropropagation (Mitras et al. 2009), and somatic embryogenesis (Capuana 2012). Other studies examined germination of cut seeds (Preece et al. 1995) and the effects of plant growth regulators on rooting and acclimatization (Lebedev and Schestibratov 2013; Fenning et al. 2022). In *Fraxinus excelsior*, further advances have been achieved through somatic embryogenesis protocols (Capuana 2012) and shoot organogenesis (Dancheva et al. 2013). Together, these studies support genetic conservation and large-scale propagation of ash species. *F. sogdiana* also has potential value in medicine, agriculture, and industry, and Aldibekova et al. (2023) highlighted its adaptive variability and possible medicinal importance. Therefore, an effective micropropagation protocol is needed for the conservation and sustainable use of this species.

Plant conservation biotechnology focuses on preserving and utilizing plant genetic resources. Among available methods, in vitro Slow Growth Storage (SGS) is an effective strategy for conserving plant biodiversity, especially rare and endemic taxa (Benelli et al. 2022; Kali et al. 2024). SGS suppresses metabolic activity, extends culture viability, and reduces subculturing, thereby lowering the risks of contamination and somaclonal variation. Commonly used compounds include Abscisic Acid (ABA) (Garcia et al. 2011; El-Dawayati et al. 2013; Trejgell et al. 2015), Chlorocholine Chloride (CCC) (Pan et al. 2014; Mitrofanova et al. 2020; Kali et al. 2024), and mannitol (Gabr and Sayed 2010; Turdiyev et al. 2020; Nurtaza et al. 2024). Osmotic agents such as mannitol and sorbitol are also used to induce osmotic stress and restrict growth further (El-Bahr et al. 2016). For *F. sogdiana*, it is therefore important to establish an in vitro conservation system that integrates micropropagation, organogenesis, and SGS.

DNA barcoding has been used since 2003 for species identification and for analyzing ecological and evolutionary relationships. A standard DNA barcode is a short (<1000 bp), variable DNA region that can be used to explore biological diversity (Kress and Erickson 2008). A comparison of seven leading plastome regions across 907 samples representing angiosperms, gymnosperms, and cryptogams showed that *rbcL*, *matK*, *trnH-psbA*, and *ITS* are generally accepted as standardized universal plant barcodes for routine applications (CBOL Plant Working Group et al. 2009). In genus *Fraxinus*, phylogenetic relationships among 40 recognized species were assessed using 106 nuclear ribosomal *ITS* sequences, and the resulting clades supported sectional delimitation with strong molecular and morphological evidence. However, combined *rps16* and *trnL-F* data contained relatively few informative sites among 15 species representing all sections (Wallander 2008).

The objectives of the present study were to develop and evaluate an effective protocol for in vitro micropropagation and SGS of *F. sogdiana*, and to determine the phylogenetic relationships of *F. sogdiana* through comparison with barcode sequences of *Fraxinus* species from the NCBI database. These approaches are expected to support conservation of genetic resources and sustainable use of this rare relict species.

MATERIALS AND METHODS

Plant material preparation and disinfection

All plant materials were collected in the Charyn State National Nature Park (43°33′55.8″ N, 79°18′17.9″ E), located in the Almaty region of Kazakhstan, under the guidance of botanists (https://charyn.kz/eng/about-the-park/). Permission to collect the endangered species was obtained from the Forestry and Wildlife Committee of the Ministry of Ecology, Geology and Natural Resources of the Republic of Kazakhstan. The mother tree is a perennial tree with a trunk diameter of 30 cm at chest level and a height of about 8 meters, which corresponds to 25-30 years of growth according to regional taxonomy tables.

The experiments were independently performed in triplicate. A total of 180 *F. sogdiana* seeds underwent a two-step surface sterilization protocol as previously described by Kali et al. (2024). In the first step, the seeds were treated with a 1.0% sodium hypochlorite solution for 30 min and then rinsed thoroughly under running tap water. In the second step, the seeds were immersed in a 5% sodium hypochlorite solution containing Tween 20 for five min. After sterilization, the seeds were rinsed three to four times with sterile distilled water, dried on sterile filter paper, and subsequently transferred to the nutrient medium for cultivation. The experiment was conducted with three independent replicates, with 10 seeds sown on each of the nutrient media MP-I to MP-VI.

Micropropagation of *Fraxinus sogdiana* plants from seeds

The sterile seeds were placed in Petri dishes containing a nutrient medium for five to seven days to soften them, making it easier to extract the embryos. The embryos were then excised from the pericarp and transferred to test tubes for further growth. Micropropagation (MP) was carried out using either Murashige and Skoog (MS) (Murashige and Skoog, 1962) mineral salt-based nutrient medium (M407, PhytoTech Labs) or Driver and Kuniyuki (DKW) (Driver and Kuniyuki, 1984) basal salt mixture (D190, PhytoTech Labs). Both were supplemented with 3% sucrose. Plant Growth Regulators (PGRs) were added to the media according to the compositions outlined in Table 1. Stock solutions of the PGRs were prepared individually and sterile-filtered through 0.22 μm filters. Then, they were added to cooled media under sterile conditions. The phytohormones α-Naphthylacetic Acid (NAA), 6-Benzylaminopurine (BAP), and Gibberellic Acid (GA_3) were each prepared at a concentration of 1 mg/mL. NAA and GA_3 were pre-dissolved in a minimal volume of 96% ethanol, and BAP was dissolved in 1 N NaOH. Once fully dissolved, the solutions were brought up to the required volume with distilled water. The pH of the medium was adjusted to 5.6-5.8 with 1 N NaOH, and 2 g/L of phytogel was added as a gelling agent. The media were then autoclaved at 121°C for 20 minutes. *F. sogdiana* cultures were maintained at 26-28°C under a 16-hour photoperiod with fluorescent lamps at an intensity of 3000 lx.

In assessing the regenerative capacity of *F. sogdiana* seeds, the initial step was to germinate the seeds on an MS medium supplemented with 0.2 mg/L of kinetin. This medium was used exclusively during the primary culture stage to obtain viable and morphologically uniform seedlings. Our study involved cultivating seedlings in six variants of medium (MP-I to MP-VI), that differed in their PGR composition (BAP, NAA, and gibberellic acid) and basal salt base. MP-I to MP-III media were based on MS salts, and MP-IV to MP-VI media were based on DKW salts. Micropropagation passages were carried out regularly every six weeks, during which morphogenetic parameters were recorded, with an average of seven to eight shoots were transferred to fresh nutrient medium per passage. For micropropagation, shoots that were visually active and morphologically normal with distinctly defined nodes were selected for further propagation.

Acclimatization

After six weeks, the *F. sogdiana* plantlets were removed from the culture vessels along with their roots. Their root systems were thoroughly rinsed with running water to remove any residual nutrient medium. Thirty seedlings were then transplanted into individual 0.5-L pots containing a sterile mixture of vermiculite and soil at a ratio of 1:1. The pots were placed under a transparent plastic dome in a misting chamber with a relative humidity of 75-85%. Over the course of two weeks, the dome was gradually raised to reduce humidity. The presence of new leaves was assessed as an indicator of survival after one month. One month later, the plants were transferred to 3-L pots filled with an autoclaved soil mixture. Complete environmental acclimatization was achieved by placing the plants in natural field conditions after six months. Primary acclimatization was conducted in a controlled environment chamber with regulated temperature and light conditions.

Slow growth storage

Slow growth storage Preservation (P) was conducted using a half-strength DKW medium supplemented with plant growth retardants, such as ABA, CCC, and mannitol. The concentrations of these retardants are detailed in Table 2. These retardants were used to modulate the growth of *F. sogdiana*. The cultures were maintained under controlled conditions for six months, including an intensity of light of 1200-1500 lux, a reduced temperature of 10°C, and a photoperiod of 16 hours of light and 8 hours of darkness. Assessments were performed biannually, during which the plant material was transferred to standard culture conditions for evaluation. Standard laboratory vessels for plant tissue culture were used: specifically, glass culture tubes PhytoTech measuring 25 × 150 mm with a round bottom. 10-15 mL of culture medium was added to each tube. Explant density was maintained at one shoot per tube. Eighteen shoots were used for each treatment variant (ABA, CCC, mannitol), with three biological replicates (six shoots per replicate).

DNA barcoding

DNA was extracted from fresh *F. sogdiana* leaves using the CTAB method (Doyle and Doyle 1987). The extracted DNA was checked for purity using 1% agarose gel electrophoresis and run at 120 V for 30 min. All of the selected samples had high-quality DNA with concentrations above 50 ng/µL. The DNA was stored in a freezer at -20°C until it was used in the next step of the experiment. The selection of universal barcode primers was guided by the relevant literature and is fully detailed in Table 3. These primers were synthesized by the Organic Synthesis Laboratory at the National Center for Biotechnology (NCB) in Astana, Kazakhstan. The amplification and sequencing methods were performed according to the standard protocol of the Plant Genetic Engineering Laboratory of the NCB (Kali et al. 2024; Sutula et al. 2024; Ramazanova et al. 2025). The PCR master mixture consisted of 40 µL, including 2 µL of genomic DNA (50 ng), 0.4 µL of 10 units Taq polymerase (Gen Lab, Widnes, UK), 4 µL of 25 mM $MgCl_2$ (Thermo Scientific, Walthem, MA, USA), 4 µL of 10X Taq buffer (Thermo Scientific, Waltham, MA, USA), 1 µL of 10 mM dNTP (Thermo Scientific), and 1 µL of forward and reverse primers (10 µmol/L stock), with the remaining volume made up with ddH_2O. PCR amplification was performed in T100 Thermal Cycler (Bio-Rad, Berkeley, CA, USA) using the following universal PCR program for all DNA regions: initial denaturation at 95°C for 5 min (one cycle), 30 cycles of denaturation at 95°C for 1 min, annealing at the optimal temperature for each primer (50-58°C; see Table 5) for 1 min and elongation at 72°C for 1 min. Finally, one cycle of 10 min was run at 72°C for extension, followed by a 4°C hold. The purified PCR products were sequenced using Sanger sequencing method using on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1. Variations of nutrient media for *Fraxinus sogdiana*

Medium	Variations*	BAP*, mg/L	NAA*, mg/L	GA_3*, mg/L
MS	MP-I	1.0	0.1	-
	MP-II	1.0	0.1	0.5
	MP-III	2.0	-	-
DKW	MP-IV	1.0	0.1	-
	MP-V	1.0	0.1	0.5
	MP-VI	2.0	-	-

Note: *MP: Micropropagation, BAP: 6-Benzylaminopurine, NAA: α-Naphthylacetic Acid, GA_3: Gibberellic Acid

Table 2. Variations in P medium with retardants for SGS of *Fraxinus sogdiana*

Variations of DKW medium*	ABA*, mg/L	CCC*, mg/L	Mannitol, mg/L
P-I	2.0	-	-
P-II	5.0	-	-
P-III	-	0.2	-
P-IV	-	0.5	-
P-V	-	-	5.0
P-VI	-	-	8.0

Note: *P: Preservation, ABA: Abscisic Acid, CCC: Chlorocholine Chloride

Table 3. Nucleotide sequences of PCR primers used for the four barcoding sequences

Primer name	Nucleotide sequence of primer	Barcoding locus	Tm (°C)	Reference
3F_KIMf	5’ - CGTACAGTACTTTTGTGTTTACGAG - 3’	*matK*	50	Zhang et al. (2012)
1R_KIMr	5’- ACCCCATTCATCTGGAAATCTTGGTTC - 3’	*matK*	50	
rbcLa_F	5’ - ATGTCACCACAAACAGAGACTAAAGC - 3’	*rbcL*	58	Kuzmina et al. (2012)
rbcLa_R	5’ - GTAAAATCAAGTCCACCRCG - 3’	*rbcL*	58	
ITS4	5’ - TCCTCCGCTTATTGATATGC - 3’	*ITS*1 and *ITS*2	55	Khan (2019)
ITS5	5’ - GGAAGTAAAAGTCGTAACAAG - 3’	*ITS*1 and *ITS*2	55	

Statistical analyses

The data representing the mean±Standard Deviation (SD) of the replicates were analyzed using MS Excel. Data on the medium type × morphological parameters, passage × morphological parameters and medium type × storage time were analyzed using Two-Way ANOVA, and a Bonferroni post hoc test was applied at a significance level of $p<0.05$. using Prism 10 software. The sequence alignment of the *F. sogdiana* species was performed using Vector NTI Advance 11.0 (Lu and Moriyama 2005), and was then used for further analyses. The nucleotide diversity score (Pi) was calculated using DnaSP v5.10 (Librado and Rozas 2009). Phylogenetic analyses were performed using the maximum likelihood method with a bootstrap procedure (10,000 replicates) in IQTree v. 3.0.1 (Wong et al. 2025), using suitable models such as TN+F+G4 for *ITS*, F81 for *rbcL* and TPM2u+F for *matK*. KU528507 for *ITS* (*Olea europaea*), MF158699.1 for *matK* (*Erysimum diffusum*) and PP969771.1 for *rbcL* (*Tanacetum vulgare*) were used as outgroups for the phylogenetic analysis. A total of 36, 28 and 16 additional sequences were retrieved from the NCBI database to be compared with our sequences and to reconstruct phylogenetic trees for *ITS*, *matK* and *rbcL,* respectively.

RESULTS AND DISCUSSION

Micropropagation of *Fraxinus sogdiana* plants from seeds

The two-step sterilization protocol ensured 100% sterility of 180 *F. sogdiana* seeds, providing to be highly effective. No microbial contamination was detected in any of the cultures throughout the in vitro cultivation period. The regenerative capacity of *F. sogdiana* seeds cultured on MS medium supplemented with 0.2 mg/L of kinetin was evaluated. Active elongation of the hypocotyl and development of the main shoot to lengths between 1.5 and 2 cm were observed by day 14 of cultivation. This initial growth enabled the establishment of viable plantlets for subsequent in vitro microclonal propagation. The composition of the culture medium significantly influenced the morphogenetic response of *F. sogdiana* explants during in vitro cultivation. Explants were cultured for six weeks on six different media (MP-I to MP-VI), which were based on MS and DKW salts (Figure 1). The DKW-based MP-V medium significantly outperformed compared the others, promoting the greatest shoot elongation, reaching 11.54 cm (Figure 1.A). This elongation was notably higher than that achieved with any other treatment, suggesting a synergistic effect of the combined PGRs in the DKW-based medium. MP-II and MP-IV supported moderate shoot elongation (6.46 cm and 6.54 cm, respectively), further highlighting the DKW medium's enhanced effect. The greater shoot elongation observed in MP-II underscores the importance of Gibberellic Acid (GA_3) in promoting shoot elongation. In contrast, the MS-based MP-III medium, supplemented with only 2.0 mg/L BAP, resulted in the lowest shoot elongation (3.16 cm). Meanwhile, MP-VI, which was supplemented with 2.0 mg/L of BAP and was based on DKW salts, slightly improved shoot elongation to 4.17 cm. These results suggest that, while a higher concentration of the BAP alone is insufficient to promote shoot growth, the presence of DKW salts provides a slight advantage.

MP-V demonstrated the highest efficiency in terms of shoot proliferation, producing an average of seven shoots per explant. This value was significantly higher than those of the other treatments, highlighting the synergistic effect of combining BAP, NAA, and GA_3 in the DKW medium. MP-II and MP-IV resulted in intermediate shoot numbers, with an average of 3.47 and 3.97 shoots per explant, respectively. MP-I, MP-III, and MP-VI exhibited the lowest proliferation rates, with an average of 2.13, 1.57, and 2.47 shoots per explant, respectively.

Further microclonal propagation of *F. sogdiana* on the MP-V medium demonstrated its effectiveness and suitability for supporting sustained growth across multiple subcultures. The MP-V medium's consistent performance over three consecutive passages highlights its potential to maintain stable morphogenetic responses, such as shoot proliferation and elongation. Figures 2 and 3 present detailed data on shoot development and multiplication dynamics during each passage. These results confirm that the explants maintained high regenerative capacity throughout the subculturing process.

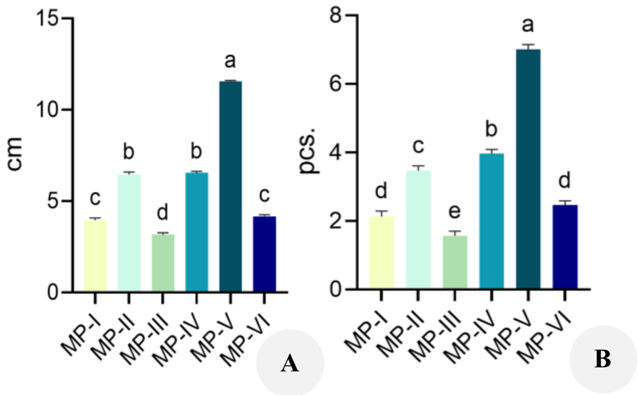

Figure 1. Effect of nutrient media composition (Table 1) on morphogenetic parameters of in vitro shoots after 6 weeks of cultivation: A. Average shoot length (cm), B. Number of shoots per explant (pcs). Data represent the mean±Standard Deviation (SD) of three independent experiments

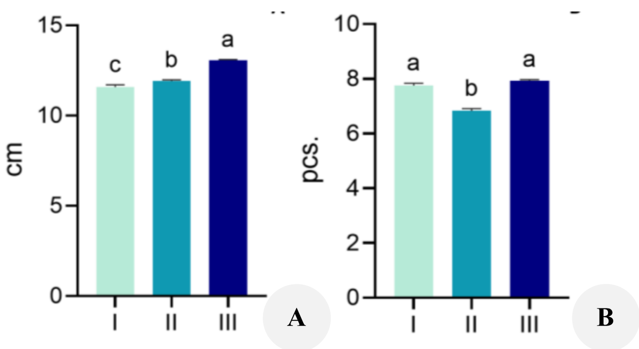

Figure 2. Micropropagation of *F. sogdiana* plants on MP-V medium across three consecutive passages: A. Average shoot length (cm), B. Number of shoots per explant (pcs). Data represent the mean±Standard Deviation (SD) of three independent experiments

An evaluation of the key morphogenetic parameters of *F. sogdiana* microshoots across three passages (I-III) revealed a consistent increase in shoot length. The maximum average length was 13.07 cm in the third passage. The modest increase was observed between the first (11.58 cm) and second (11.92 cm) passage. The most pronounced elongation occurred between the second and third passages. This suggests that growth processes were stimulated more actively during prolonged in vitro cultivation. The number of shoots per explant fluctuated across passages, decreasing to an average of 6.83 shoots in the second passage before increasing to an average of 7.93 shoots in the third passage. This fluctuation is likely associated with the explant's adaptation to the in vitro culture. Overall, the observed growth dynamics highlight the effectiveness of the MP-V medium in supporting the stable development of shoots and the robust multiplication of *F. sogdiana* through successive subcultures (see Figure 3).

Acclimatization of *Fraxinus sogdiana* plants

One hundred percent rhizogenesis was achieved in all cultivated microshoots on the MP-V medium. The well-rooted *F. sogdiana* plantlets were transferred to 0.5-L pots containing a sterile 1:1 ratio of vermiculite and soil for growing. After six weeks, the plantlets were transplanted into soil. One month later, the acclimated plants were moved outdoors. All ten plants demonstrated a 100% survival rate over a six-month period under ex vitro conditions. Subsequently, the plants were permanently planted in the Astana Botanical Garden for further observation under natural conditions. Figure 4 illustrates the stages of *F. sogdiana* acclimatization following transfer from in vitro culture.

In vitro preservation of plants by slow growth storage

The data in Figure 5 demonstrate the effects of growth inhibitors on in vitro shoot development during storage over two, four, and six-month periods. Abscisic Acid (ABA), Chlorocholine Chloride (CCC), and mannitol produced different responses in shoot elongation across six media treatments with various concentrations of these growth retardants. A gradual increase in shoot length was observed with rising concentrations of ABA and CCC. Specifically, shoot length in ABA-treated media increased from 0.38 cm to 0.55 cm as the ABA concentration increased.

Similarly, CCC treatments resulted in a significant increase in shoot length, ranging from 0.88 cm to 0.96 cm. The greatest shoot elongation occurred in media supplemented with CCC, particularly at a concentration of 0.5 mg/L. Conversely, both ABA and mannitol were associated with slower, more inhibited shoot development. These results suggest that CCC is the most effective growth inhibitor for maintaining viability while preventing excessive growth during in vitro storage. In contrast, supplementation with mannitol, particularly at concentrations of 5 and 8 mg/L, significantly reduced shoot growth.

After six months of SGS, the same plants were transferred to an MP-V medium containing 1.0 mg/L BAP, 0.1 mg/L NAA, and 0.5 mg/L gibberellic acid. Following this transfer, the plants successfully resumed growth and development, as evidenced by the complete restoration of vegetative activity in the tested samples (Table 4). According to our data, using CCC at concentrations of 0.2 and 0.5 mg/L ensured 94.4% and 100% plant recovery, respectively, after the in vitro slow growth phase. Conversely, ABA treatment decreased survival rates from 66.7% to 44.4% as the concentration increased. Mannitol was the least effective, with survival rates of 38.9% and 22.2%, respectively.

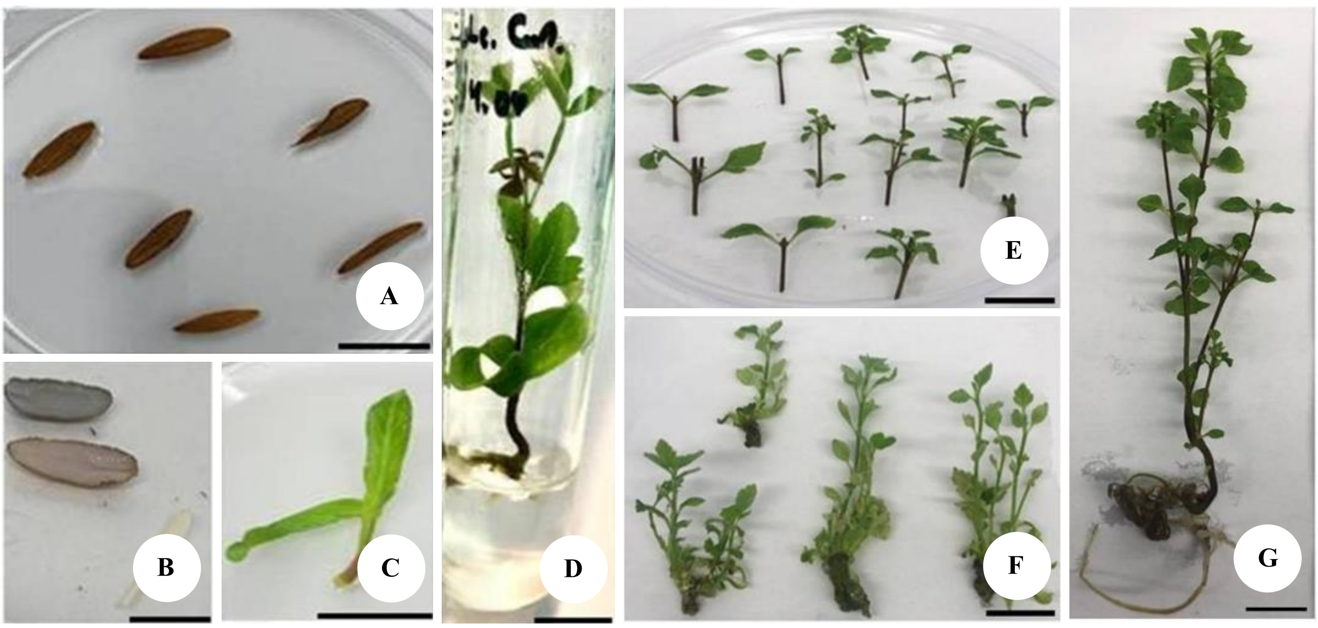

Figure 3. Micropropagation of *Fraxinus sogdiana*: A. Seeds, B. Embryo extraction from the seed, C. Shoot on day 3 of cultivation, D. Plant at the fourth week of cultivation, E. Microcuttings, F. Development of *Fraxinus sogdiana* shoots in in vitro culture, G. Six weeks old plants. Scale bar 1 cm (A-F) and 1.5 cm (G)

Figure 4. *Fraxinus sogdiana* plants under ex vitro conditions. A. Rooted microplants transferred to pots containing a 1:1 mixture of vermiculite and soil, B. Acclimatized plants after six months of cultivation, and C. Plants established in open ground

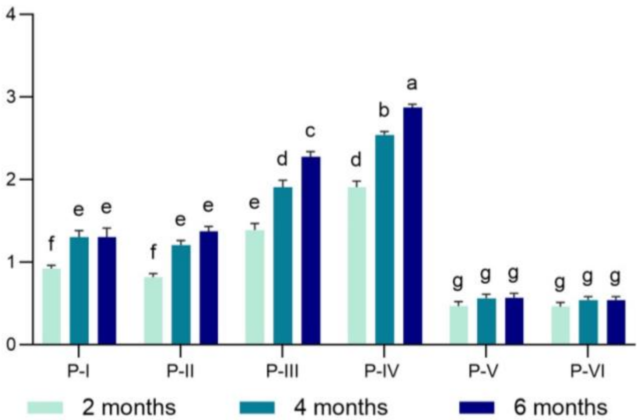

Figure 5. Shoot development in length (cm) during two, four and six months of slow growth on P I-VI medium. Variations in P medium (P I-VI) were given in Table 2. Data represent the mean±Standard Deviation (SD) of three independent experiments

Thus, the results obtained highlight the potential of using CCC as an effective retardant for the slow growth of *F. sogdiana* in vitro, followed by the successful recovery of growth and development. Selecting an appropriate growth regulator is essential for optimizing slow growth protocols and preserving the genetic resources of rare and valuable plant species.

Phylogenetic analysis

In this study, phylogenetic analysis was performed using three barcoding markers to identify the phylogenetic relationships and species of *F. sogdiana* plants. *ITS*, *matK* and *rbcL* gene sequences from these plants were analyzed alongside recall gene sequences from *Fraxinus* species in the NCBI database to infer their phylogenetic relationships. After alignment, the lengths of the sequences were determined to be 480 bp for *ITS*, 719 bp for *matK,* and 513 bp for *rbcL*. Monomorphic sites spanned 376, 508, and 688 bp, while polymorphic sites ranged from 5 to 103 bp. Genetic variation in the sequences was assessed using two neutrality tests: nucleotide diversity (Pi) and Tajima's neutrality test (D). The Pi values ranged from 0.00134 to 0.04609, and the D values from 0.001340 to 0.23393 (Table 5). The *ITS*, *matK*, and *rbcL* sequence accessions for *F. sogdiana* (PP830058, PP836055, and PP836056, respectively) are available in the NCBI database.

A phylogenetic analysis of *Fraxinus* species was conducted, comparing *F. sogdiana* from Kazakhstan with 36 species from the NCBI. The analysis categorized the species into five main groups based on their *ITS* region patterns. The species were clearly divided into groups according to the five sections of the genus *Fraxinus*: *Fraxinus*, *Sciadanthus*, *Melioides*, *Pauciflorae,* and *Ornus*. The average pairwise distances were 0,028, 0,024 0,016, 0,026, 0,014, respectively. Notably, seven sequences of the *Fraxinus angustifolia*, *Fraxinus obliqua*, *F. sogdiana*, and *Fraxinus turkestanica* species, which are considered synonyms, were grouped together. The branches corresponded to 89% support (Figure 6), and polymorphic sites were found (Table 6).

The phylogenetic analysis categorized *Fraxinus* species into four main groups based on their *matK* gene patterns. The first group included 11 species from the *Ornus*, *Sciadanthus*, and *Dipetelae* sections. The second and third groups mainly comprised *Fraxinus* species. The fourth group consisted of species from the *Fraxinus* and *Melioides* sections. The main finding was that species from China and Kazakhstan were clustered together in the *matK* gene region with a high bootstrap value of 97. A phylogenetic analysis of *Fraxinus* species divided them into two main groups based on the *rbcL* gene patterns. The first group contained species from the *Ornus* and *Fraxinus* sections. The second group included a mix of species from the *Ornus*, *Fraxinus*, and *Sciadanthus* sections.

Discussion

The DKW-based MP-V medium supplemented with 1.0 mg/L BAP, 0.1 mg/L NAA, and 0.5 mg/L GA_3 promoted shoot elongation more effectively than the MS-based medium. The synergistic effect of gibberellic acid with BAP and NAA appears to play a key role in enhancing shoot development in enhancing shoot development by stimulating cell division and elongation. In contrast, media with a high BAP concentration (2.0 mg/L), such as MP-III and MP-VI, resulted in the poorest shoot growth. These results demonstrate that, although BAP is an effective cytokinin, its application at high concentrations is not recommended, as excessive stimulation of cell division without sufficient elongation can inhibit overall shoot development. Furthermore, the growth dynamics observed over three successive passages on the MP-V medium confirm the efficacy of the hormonal combinations used. The consistent increase in shoot length and number across passages indicates stable morphogenic performance and adaptation to in vitro conditions. Overall, these results underscore the importance of both the basal nutrient composition and specific growth regulator combinations in optimizing the in vitro shoot development of *F. sogdiana*. The superior performance of DKW-based media, particularly MP-V, highlights their suitability for efficient micropropagation protocols of this rare species. DKW was the most effective at promoting shoot growth, likely due to its unique mineral composition. Our findings are consistent with previous reports. For example, Hammatt (1994) reported successfully establishing axillary shoot proliferation from adult *F. excelsior* on a DKW medium supplemented with 22 µM BAP, and (Pérez-Parrón et al. 1995) achieved the highest shoot proliferation (5.3 shoots per mature explant) using shoot tips and nodal explants from both mature and juvenile *F. angustifolia* on a DKW medium supplemented with 4.4 µM BA and 0.98 µM IBA. The rooting response observed in all microshoots on the MP-V medium suggests a potential synergistic effect of the phytohormonal combination used. Auxin (NAA) facilitates root initiation, and the combination of auxin and BAP promotes cellular proliferation. Additionally, gibberellic acid contributes to cell elongation and differentiation, supporting the overall regeneration process.

Table 5. Aligned sequence features of the candidate DNA barcoding markers

	ITS	***matK* gene**	***rbcL* gene**
Original sequence length (bp)	469	747	513
Total aligned length (bp)	480	719	513
GC content (%)	60.9	34.1	43.4
Codon count	-	239	171
Number of monomorphic sites	376	688	508
Number of polymorphic sites	103	15	5
Total number of InDels sites	36	16	0
Overlapping InDels sites	10	13	0
Number of singleton variable sites	22	8	2
Total number of mutations (Eta)	93	15	5
Parsimony informative sites (PIC)	81	7	3
Nucleotide diversity (Pi)	0.04609	0.00310	0.00134
Tajima's neutrality test (D)	0.23393	0.004009	0.001340
Mean nucleotide difference (k)	20.464	2.182	0.687
Number of Haplotypes (h)	31	11	6
Haplotype diversity (Hd)	0.979	0.837	0.506
Variance of Haplotype diversity	0.00017	0.00195	0.01132
Sequence conservation (C)	0.784	0.976	0.990
Conservation threshold (CT)	0.88	1	1

Table 4. Survival rate of *Fraxinus sogdiana* plants after 6 months of slow growth storage

P medium with retardants for SGS		**Number of plants transplanted to a medium with retardants, pcs.**	**Number of plants after 6 months of storage, pcs.**	**Number of plants regenerated after one month of cultivation, pcs.**	**Survival rate, %**
ABA	2.0 mg/L	18	15	12	66.7
	5.0 mg/L	18	12	8	44.4
CCC	0.2 mg/L	18	17	17	94.4
	0.5 mg/L	18	18	18	100
Mannitol	5.0 mg/L	18	12	7	38.9
	8.0 mg/L	18	10	4	22.2

Table 6. Polymorphic sites of the *ITS* sequences alignment among *Fraxinus* species that were previously considered synonyms

Species	**Accession number NCBI**	**Countries**	**Polymorphic sites#**					
			54	**291**	**314**	**392**	**459**	**469**
Fraxinus sogdiana Bunge*	PP830058	Kazakhstan	C	C	G	C	C	C
Fraxinus sogdiana Bunge	HQ705287		C	C	A	C	C	C
Fraxinus turkestanica Carrière	HQ705302		C	C	G	T	C	T
Fraxinus angustifolia Turkey Vahl	EU314817	Turkey	C	C	G	T	C	C
Fraxinus angustifolia Vahl	EU314816	Israel	C	C	G	T	C	C
Fraxinus angustifolia subsp. *syriaca* (Boiss.) Yalt.	HQ705298		G	C	A	C	T	T
Fraxinus obliqua Tausch	HQ705255		C	T	G	T	C	C

Note: *The species in this study. #A: Adenine, C: Cytosine, T: Thymine, G: Guanine

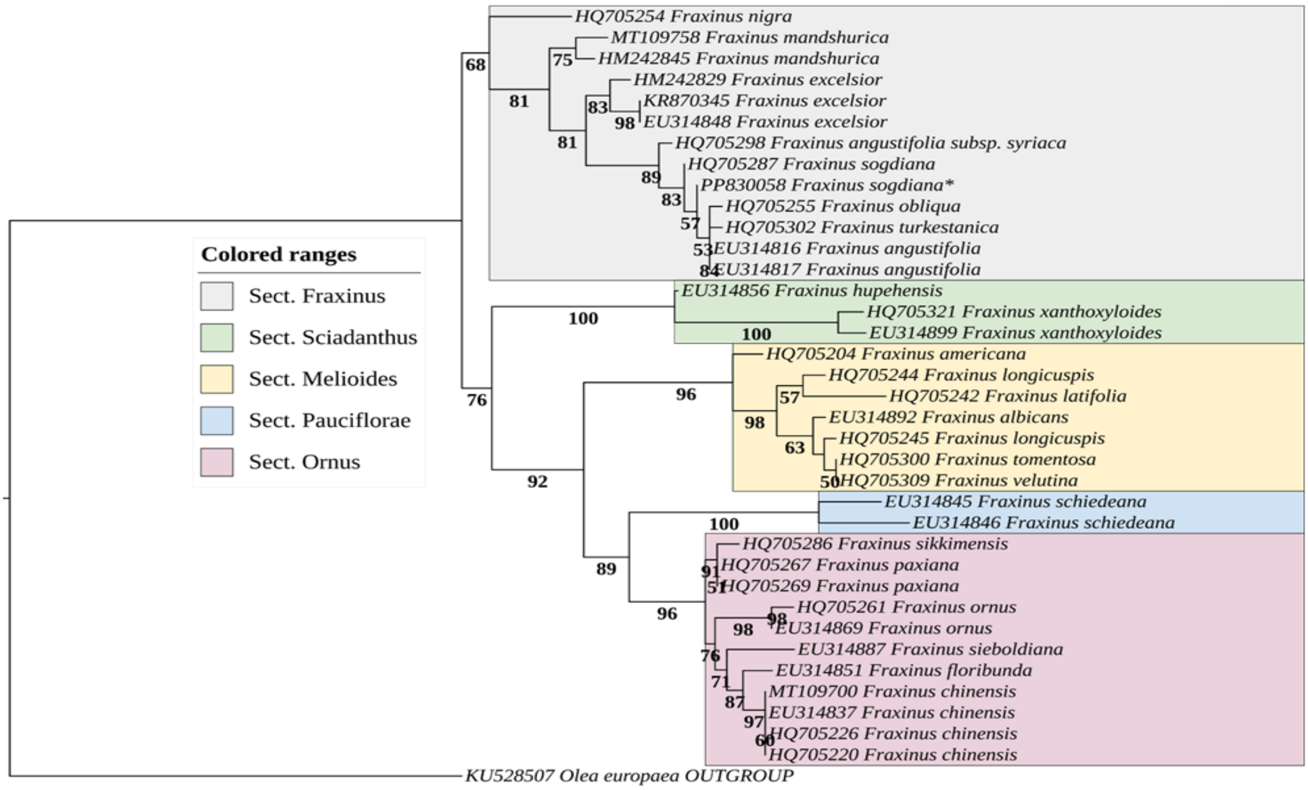

Figure 6. The phylogenetic tree of *Fraxinus* species based on the *ITS* region sequence using the maximum likelihood method. Numbers above branches indicate bootstrap values. *The species in this study

The slow growth storage technique is an effective in vitro method for sustainably preserving plant species intended for conservation and future use in a sustainable manner. This approach optimizes storage space and reduces maintenance costs by regulating the growth and development of plantlets (Cremon et al. 2018; Benelli et al. 2022). Several factors influence the successful application of SGS, including temperature, light or dark conditions, and the composition of the culture medium. Critical medium components include mineral and sucrose concentrations, as well as the presence or absence of PGRs, osmotic agents, and growth inhibitors (Trejgell et al. 2015).

In the present study, variants P-I and P-II of the medium, supplemented with 2.0 and 5.0 mg/L ABA, respectively, effectively suppressed shoot growth, as evidenced by minimal shoot elongation. Garcia et al. (2011) reported a similar inhibitory effect of ABA on in vitro conservation in *Passiflora suberosa*, with a 50% decrease in survival rate accompanied by leaf abscission and the formation of numerous thick roots. However, studies on *Senecio macrophyllus* (Trejgell et al. 2015) and *Phoenix dactylifera* (El-Dawayati et al. 2013) demonstrated that ABA supplementation enhances shoot survival and proliferation during post-storage recovery compared to shoots maintained on ABA-free media. Under SGS conditions, *F. sogdiana* exhibited the most favorable shoot development on media supplemented with CCC at concentrations of 0.2 and 0.5 mg/L. These results align with previous research. For example, SGS studies have reported positive outcomes. For instance, CCC concentrations ranging from 0.2 to 0.4 mg/L have been shown to have positive outcomes in media used for *Chrysanthemum* and *Syringa* (Mitrofanova et al. 2020). A residual effect of CCC was also noted in *Euonymus koopmannii*, where an initial concentration of 0.5 mg/L improved plant performance after six months of storage (Kali et al. 2024). Similarly, Pan et al. (2014) identified CCC as a key component in the successful slow-growth conservation of *Vitis heyneana*. They obtained optimal results using a medium supplemented with CCC (5.0 g/L) and mannitol (10 g/L), as well as by modifying environmental factors such as light intensity and gas exchange using air-permeable films (Pan et al. 2014). In the present study, shoot elongation was significantly reduced in media P-V and P-VI, which contained 5.0 and 8.0 mg/L of mannitol, respectively. These results are consistent with previous reports showing the effectiveness of mannitol in inducing slow growth in various plant species (El-Bahr et al. 2016; Nurtaza et al. 2024). The effectiveness of different growth retardants for SGS may vary depending on the plant species. This suggests that species-specific responses must be considered when designing in vitro storage protocols.

This study investigated the phylogenetic relationships and species identification of *F. sogdiana* using three standard DNA barcoding markers, including one nuclear *ITS* region and two chloroplast *matK* and *rbcL* genes. Our results indicated that the genetic variation and neutrality tests among the species according to the *ITS* region revealed high interspecific variation within the *Fraxinus* genus. Notably, seven sequences of the *F. angustifolia*, *F. obliqua*, *F. sogdiana*, and *F. turkestania* species, which are considered synonyms, were grouped together. The Bohemian botanist Ignaz Friedrich Tausch (1793-1848) primarily considered *F. obliqua* Tausch a synonym of *F. excelsior* subsp. *coriariifolia* (Govaerts 1996) and linked to *F. angustifolia* subsp. *oxycarpa* (Bussmann et al. 2025). Despite the reliable results in this work, further research is required to more accurately determine the taxonomic relationship of *F. obliqua*, based on multi-locus sequences. Eva Wallander also supported the idea of sections differentiated by *Fraxinus* species barcoding markers, including the *ITS* region, as well as morphological characteristics (Wallander 2008). We found limited interspecific variation according to *matK* and *rbcL* gene sequences, and *Fraxinus* populations are relatively genetically uniform. The main finding is that the Kazakhstan and Chinese populations cluster together with high support (*matK*), suggesting a common haplotype and genetic uniformity. These findings contribute to the development of effective conservation strategies and the preservation of genetic resources for *F. sogdiana* and other rare plant species, while establishing a valuable foundation for future biotechnological and molecular research.

In conclusion, this study evaluated in vitro micropropagation and SGS conditions for the rare and relict species *F. sogdiana*. The results highlight the effectiveness of optimized culture media, particularly the DKW-based MP-V medium, in promoting shoot proliferation and growth. Additionally, applying specific growth retardants, such as CCC, under slow-growth conditions enabled successful medium-term storage by balancing plant survival and suppression of excessive growth. These results provide important insights into the physiological responses of *F. sogdiana* in in vitro and establish a reliable foundation for its efficient micropropagation and conservation. The developed protocols can be adapted to propagate and preserve other rare and endangered plant species, thereby supporting broader ex situ conservation initiatives. Phylogenetic analysis based on the *ITS* marker clarified sectional relationships within the *Fraxinus* species and revealed strong genetic clustering among previously synonymous species. Additionally, the *matK* and *rbcL* markers demonstrated a close genetic affinity between the *F. sogdiana* species from Kazakhstan and China, despite the limited taxonomic resolution of the *matK* and *rbcL* markers.

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