



Restoring populations of *Populus pruinosa* Schrenk through biotechnology

Timur Turdiyev^{1,2} · Irina Kovalchuk^{1,2} · Aigul Madenova¹ · Angsagan Kuan^{1,3} · Natalya Mikhailenko^{1,2} · Balnur Kabylbekova¹ · Saule Baizhumanova¹ · Kamila Yemesheva¹ · Zakir Tuigunov² · Izbassar Rakhimbayev²

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Abstract

The most effective method to combat desertification is planting artificial forests of *Populus pruinosa*. In the Zhetysu and Almaty regions, samples were gathered from natural stands, which *P. pruinosa* and *P. diversifolia* likely displaced. The morphological attributes of 27 samples were related to one species, but one sample was causing doubt. PCR analyses were performed using eight microsatellite markers. The genotyping results revealed that all of the 28 samples collected were of the same type of *P. pruinosa*. They can effectively be introduced into *in vitro* culture in winter when physiological dormancy has ended. Annual lignified cuttings 6 to 7 cm long with 1 to 2 buds from selected plus trees were sterilized from saprophytic microflora by bleach NaOCl (1:1) for 10 min and 0.1% HgCl₂ solution for 5 min and placed on the medium. Both male and female plants were propagated in the medium that contained ½ Murashige and Skoog (MS), 0.1 mg L⁻¹ BAP; 0.02 mg L⁻¹ GA; 20 g L⁻¹ Glucose; pH 5.7, where the multiplication rate averaged 3.8 to 4.5, and the shoot length at 5.7 to 7.0 cm in 4 to 6 wk. For the transferring regenerated plants to soil in pots, the best substrate was found as peat, humus, and sand at a ratio of 50:40:10, respectively.

Keywords *P. pruinosa* · Aseptic plants · Clonal micropropagation · Rhizogenesis · Pot plant

Introduction

The only 5% territory of Kazakhstan is covered by forests. The main constraints to forest expansion are the aridity of the climate and the susceptibility to desertification of a large part of the territory (about 60%). Drought-resistant and salt-tolerant species of woody plants are used to combat desertification. One of them is turanga poplar, which can grow on sandy, saline, and depleted soils.

There are three species of turanga poplars in Kazakhstan: *Populus pruinosa* Schrenk, *Populus diversifolia* Schrenk,

and *Populus litwinowiana* Dode (Besschetnov and Grudzhinskaya 1981). Paleobotanical studies indicate its origin and flourishing in the Miocene era 23 to 5.3 million yr ago (Kornilova 1966). Fossilized remains found in various regions of Kazakhstan prove that this species had a wide range even with strong aridization of the climate in the Pliocene era 5.3 to 2.6 million yr ago (Kutuzkina 1957, 1976; Vykholdtsev and Makulbekov 1987).

P. pruinosa is listed in the Red Book of Kazakhstan (Baitulin 2014). In 2007, it was included in the International Red Book as an endangered species (IUCN 2007). Currently, only a few populations that are highly regressive have been able to survive. The main reason for this is the breakdown of the demographic structure due to gender imbalance, leading to a reduction in numbers and, consequently, population density.

In Kazakhstan, Turanga poplars are found mainly in the floodplains of the Ili and Syr Darya rivers. Sometimes there are places where the two species—*P. pruinosa* and *P. diversifolia*—grow in mixed form (Dimeyeva *et al.* 2023). The two species have morphological differences in their leaves. *P. diversifolia* has two types of leaves: juvenile leaves, which are narrowly elongated and leaves on mature shoots, which

✉ Angsagan Kuan
kuan.angsagan2001@gmail.com

¹ Laboratory of Plant Microclonal Propagation, Kazakh National Agrarian Research University, Almaty 050010, Kazakhstan

² Laboratory of Germplasm Cryopreservation, Institute of Plant Biology and Biotechnology, Almaty 050040, Kazakhstan

³ Department of Biotechnology, Faculty of Biology and Biotechnology, Al-Farabi Kazakh National University, Almaty 050040, Kazakhstan

are usually wider, rhombic, or ovoid. The leaves of *P. pruinosa* are ovate, rounded, and broad ovoid (Orwa *et al.* 2009; Zhai *et al.* 2022).

Turanga poplar is a dioecious plant that flowers heterosexually, making cross-pollination difficult in low population density when male and female individuals are located far apart. Artificial vegetative propagation by cuttings is very difficult due to the too-low rooting capacity of both stem and root cuttings even when using various rhizogenesis stimulators (Radionenko 1945; Ratkovsky 1948; Fortunatov 1949; Bogdanov 1965; Besschetnov and Grudzinskaya 1981). In addition, damage to plants of rare endangered species in the natural ecosystem is legally prohibited (Baitulin 2014).

In the geological past, *P. pruinosa* had an extensive range, as evidenced by the results of paleobotany studies conducted in Kazakhstan (Kornilova 1966; Baitenov 1979). Over time, this species has become a paleoendemic with a rapidly decreasing range, currently represented in only a few regressive populations. Meanwhile, the threat of extinction of this species has been repeatedly reported by many scientists since the last century (Besschetnov and Grudzinskaya 1981; Bykov 1966). However, the area continues to shrink to a critical level and the degradation of remaining populations is accelerating.

The potential for the species to be used in protective forest plantations and greening human settlements in arid regions has been investigated by conducting studies on growing conditions, breeding patterns, and potential (Besschetnov and Grudzinskaya 1981). It was pointed out that the decrease in riparian forests was linked to changes in hydrological systems, a decrease in the amount of groundwater, fires, and the use of livestock grazing. After this species was included in the Red Book of Kazakhstan (Baitulin 2014; Bykov 1981), efforts were primarily focused on the conservation of the species in natural reserves and obtaining planting material using *in vitro* propagation. The current state of turanga stands in southeastern Kazakhstan (Almaty region) was assessed (Rakhimzhanov *et al.* 2021), which showed a predominance of mostly mature and over-mature trees. Natural regeneration in the form of root individuals was observed only in some small areas, characterizing plantings as unstable.

The need to solve the problem of breeding and conservation of *P. pruinosa* gene pool in Kazakhstan was brought to the attention of international environmental organizations. For example, a UNDP (United Nations Development Program) grant was allocated to the Republican Forest Breeding Center of the Forestry Committee under the project “Biodiversity in Kazakhstan” (Agazhayeva 2018).

Another example is connected with the activity of the Public Organization “Balkhash Ecological Center,” which executed the project “Biodiversity of northern Balkhash region. Preservation and restoration of turanga groves.”

The project is supported by the Small Grants Program of the Global Environmental Fund. *P. pruinosa* populations are highly regressive and urgent rescue measures are required (Pustlaikin 2001).

It should be noted that riparian forests provide necessary nutritional, regulating, and cultural ecosystem services. They have a significant habitat-forming, soil-strengthening, and reclamation value, for example, they generally stabilize the ecological situation in floodplains in arid regions (Rakhimzhanov *et al.* 2021; Thomas and Lang 2021).

In the natural environment, this species breeds with seeds, but for a number of reasons, they do not thrive well in harsh environmental conditions, resulting in population regression. The species is also capable of vegetative propagation, which makes it possible to apply clonal micropropagation, choosing the best trees characterized by high growth and development rate and selecting conditions for *in vitro* cloning.

The study of genetic information is important for species determination and, as a consequence, the selection of samples for further reproduction. The genetic diversity of *P. pruinosa* is due to geographical areas of origin, cultivation, and targeted selection. Confirming the validity of accessions, and their lineage, is basic to the development of molecular selection approaches and the establishment of a collection of accessions with optimal representation of biodiversity. Our goal was to classify the turanga population in the floodplains of the river Ili by describing genetic diversity and to compare the morphology and reflectivity of leaves of the proposed parent species and plantations in the Almaty and Zhetysay regions.

To recover declining populations of *P. pruinosa* in the floodplains of the Ili and Syr Darya rivers, the most logical strategies for clonal micropropagation and growing seedlings in pots with closed root systems were determined.

Materials and Methods

Study Objects The object of the study was to restore the population of *P. pruinosa* growing in Kazakhstan in the floodplains of the Ili River (Dimeyeva *et al.* 2023). GPS device (Garmin Etrex SE) was used to record the coordinates of the place of plant material collection (N45°03.355', E075°17.559'; N44°52.846', E075°54.884'; N43°46.864', E080°06.774'; N43°46.264', E080°19.539'; N43°47.610', E075°10.789'; N43°44.543', E073°51.133'; N43°44.562', E073°51.127'; N43°44.581', E073°51.121'; N43°50.418', E080°27.739'; N43°50.415', E080°76.761'; N43°50.403', E080°27.753'; N43°50.416', E080°27.767'; N43°50.505', E080°27.324'; N43°50.429', E080°27.917'; N43°50.191', E080°25.986'; N43°50.177', E080°25.966'; N43°48.339', E080°26.658'; N43°48.317', E080°26.656'; N43°47.888', E080°27.192'; N43°47.884', E080°27.184'; N43°48.984',

E080°24.582'; N43°48.899', E080°24.555'; N43°50.539', E080°03.825'; N43°50.536', E080°03.822'; N43°49.597', E080°01.668'; N43°49.000', E079°59.842'; N43°52.647', E079°27.594'; N43°45.680', E078°57.348').

DNA Extraction and SSR Marker Analysis DNA extraction was conducted by the protocol CTAB (Doyle and Doyle 1987) with modifications (Madenova *et al.* 2024) from green fresh leaves of 28 samples of *P. pruinosa*, which were collected from two regions of Kazakhstan (Zhetysu and Almaty). The DNA concentration in each sample was measured using agarose gel (1.5%) and later confirmed with NanoDrop (TM 2000/2000c ND-2000, Thermo Fisher Scientific Inc., Waltham, MA). Before the PCR, the samples were kept at -25°C and had a final concentration of $5.0\text{ ng }\mu\text{L}^{-1}$. Genetic diversity in the collected samples was determined using eight microsatellite SSR markers and the amplification mode was optimized for *P. pruinosa* for PCR analysis (Table 1.). The amplified fragments were subjected to electrophoresis on a 1.5% agarose gel (w/v) using $0.5\times$ TBE buffer at a stable voltage of 80 V for 100 min. The gel staining was performed with ethidium bromide and graphical images were obtained using a UV Gel Doc system (omniDOC Gel

Documentation System, Cleaver Scientific Ltd, Rugby, UK). A DNA ladder gene of 100 bp in length was used as a molecular mass marker.

Data Analysis The data analysis was based on the molecular analysis results. Electrophoresis was used to visualize PCR products. Each amplified PCR product had its band pattern scored as '1' for polymorphism, '0' for the absence of polymorphism, and 'n/a' for no data. Genotype data was determined by analyzing stained gels and using Popgene software (1.32 version) (Yeh *et al.* 2000). The genetic diversity score of the genotype was calculated through Popgene (1.32 version). In addition, Popgene (version 1.32) (Yeh *et al.* 2000) has been used to estimate various indices of genetic diversity, such as the effective number of alleles (Ne), Shannon's information index (I), and Nei's gene diversity (h). The polymorphism information content (PIC) was calculated for each primer according to the criteria proposed by Baloch *et al.* (2015). Population genetic estimation was carried out with the help of STRUCTURE software (version 2.3.4) (Pritchard *et al.* 2000), and the analysis was carried out by calculation of coefficient deltaK through STRUCTURE HARVESTER (Earl and Bridgett 2012).

Table 1. An overview of the tested *Populus Pruinosa* Schrenk simple sequence repeats (SSRs) markers

Primer	Forward/reverse primer	Bases	MP*	Group	Length (bp)	Program	References
WPMS 14	GATCCTGAACATGTCGT ACTTC ACGATAACCTGCGAGAAA TGT	22	72.2	1	245	1 cycle (94°C 3 min), 30 cycles (94°C 45 s, 60°C 45 s, 72°C 105 s), 1 cycle (72 °C 10 min)	Van der Schoot <i>et al.</i> 2000
WPMS 20	GTGCGCACATCTATGACT ATCG ATCTTGTAATTC TCCGGGCATCT	22	64.4	2	204–235	1 cycle (94°C 15 min), 30 cycles (94°C 45 s, 56°C 45 s, 72°C 105 s), 1 cycle (72°C 10 min)	Komárková <i>et al.</i> 2020
PMGC** 2679	GGAATCCGTTTAGGGATC TG CGTCTGGAGAACGTGATTAG	20	62.2	2	90–110		Mashkina <i>et al.</i> 2016
PMGC 433	GCAGCATTTGTAATAAT AAAAG AAGGGGTCTATTATCCACG	23	56.5	3	190–210	1 cycle (94°C 3 min), 35 cycles (94°C 30 s, 55°C 40 s, 72°C 20 s), 1 cycle (72°C 5 min)	Blonder <i>et al.</i> 2020
WPMS 5	TTCTTTTCAACTGCCTA ACTT TGATCCAATAAC AGACAGAACA	22	58.6	3	290–320		Van der Schoot <i>et al.</i> 2000
PMGC 2852	ATAATCTCCCTAGCTTAA TTCC GAATAACATGGATAATGT GTTTG	22	56.2	3	90–120		Mashkina <i>et al.</i> 2016
PMGC 2571	TCTCGCAGATTCATGTAA CCC GACTGTATGTTGACC ATGCC	21	63.9	4	84–120	1 cycle (94°C 3 min), 35 cycles (94°C 30 s, 56°C 40 s, 72°C 20 s), 1 cycle (72 oC 5 min)	Flesher <i>et al.</i> 2016
PMGC 2060	CTCTCAAATGCTGATTTA CCG TCTTCAGTTGCGAGTA TTCAAAG	21	61.2	5	140–150	1 cycle (94°C 15 min), 30 cycles (94°C 45 s, 54°C 45 s, 72°C 105 s), 1 cycle (72°C 10 min)	Mashkina <i>et al.</i> 2016

*MP - Melting Point, **PMGC - Poplar Molecular Genetics Cooperative

Study objects and methods of microclonal propagation The object of study for developing a protocol for microclonal propagation of *P. pruinosa* was annual single-tree cuttings at the winter dormancy stage collected from male and female trees. The sex of the trees was determined in spring at the flowering stage (Fig. 1).

Table 2. shows the locations of the collection sites for microclonal propagation studies.

Obtaining aseptic plants was achieved by cutting annual single-tree cuttings 20 to 25 cm long from selected plus trees of *P. pruinosa* in winter after the physiological dormancy had been completed. *In vitro* inoculation was carried out in two ways: (1) lignified cuttings were cut into segments 6 to 7 cm long with 2 to 3 buds, sterilized, and planted on nutrient medium; and (2) shoots from lignified cuttings were germinated, divided into segments with one bud, sterilized, and planted on Murashige and Skoog (MS; Murashige and Skoog 1962) medium. In both versions, saprophytic microflora was sterilized with bleach NaOCl (1:1) for 10 min and with a 0.1% HgCl₂ solution for 5 min and washed three times with sterile distilled water (Turdiyev *et al.* 2024).

To detect internal systemic infections, the basal part of shoots was planted in Petri dishes with screening nutrient medium 523 (Viss *et al.* 1991) containing 10.0 g L⁻¹ Sucrose, 8.0 g L⁻¹ Casein hydrolysate, 4.0 g L⁻¹ Yeast extract, 2.0 g L⁻¹ KH₂PO₄, 15.0 g L⁻¹ MgSO₄•7H₂O, 6.0 g L⁻¹ Gelrite. Cultures were incubated for 1 to 3 wk at 23 to 25°C (Singh *et al.* 2013).

To obtain aseptic plants, 10 to 15 mm small cuttings were placed in tubes with Murashige and Skoog (MS) nutrient medium with growth regulators: 6-benzylaminopurine (BAP) at a concentration of 0.3 mg L⁻¹ and β-indolyl-3-butyric acid (IBA) at a concentration of 0.01 mg L⁻¹. After

Table 2. Coordinates of *P. pruinosa* plant material collection site

Collection point number	Tree sex	GPS coordinates	Height at elevation, m
1	Male	N 43°44'33.26" E 073°51'07.80"	497
2	Male	N 43°44'33.06" E 073°51'08.40"	493
3	Male	N 43°44'33.32" E 073°51'07.81"	496
4	Male	N 43°52.647' E 079°27.594'	509
5	Male	N 43°52.654' E 079°27.611'	498
6	Male	N 43°45'41.05" E 078°57'20.39"	486
7	Female	N 43°45'41.05" E 078°57'20.39"	486
8	Female	N 43°45'41.05" E 078°57'20.39"	486

leaf formation, the small cuttings were transplanted on the nutrient media of different compositions for growth and propagation:

1. ½ Murashige and Skoog (MS), 20.0 g L⁻¹ Glucose; pH 5.7
2. MS, 0.1 mg L⁻¹ 6-benzylaminopurine (BAP); 0.02 mg L⁻¹ Gibberellic acid (GA); 30.0 g L⁻¹ Sucrose; pH 5.7
3. Woody Plant Medium (WPM), 0.1 mg L⁻¹ BAP; 0.02 mg L⁻¹ GA; 20.0 g L⁻¹ Glucose; pH 5.7
4. Driver and Kuniyuki Walnut (DKW) medium, 0.5 mg L⁻¹ Thiamin; 0.1 mg L⁻¹ BAP; 0.02 mg L⁻¹ GA-0.02; 20.0 g L⁻¹ Glucose; pH 5.7



(a)



(b)



(c)

Figure 1. Flowering of *Populus pruinosa* Schrenk trees: a flowering of male trees; b flowering of female trees; c formation of female ovaries.

5. MS, 0.1 mg L⁻¹ BAP; 0.02 mg L⁻¹ GA; 20.0 g L⁻¹ Glucose; pH 5.7
6. ½ MS, 0.1 mg L⁻¹ BAP; 0.02 mg L⁻¹ GA; 20.0 g L⁻¹ Glucose; pH 5.7.

Monthly observations and records were made, and the state and number of shoots formed were noted. The average multiplication rate per passage was calculated for each genotype:

$$Mr = a/b \cdot c, \quad (1)$$

where a is the number of new shoots, b is the number of shoots planted for propagation, and c is the number of passages.

Propagated mini cuttings were transplanted onto MS or ½ MS each containing 0.5 mg L⁻¹ IBA to promote root formation.

The media were sterilized in an autoclave (UTKBS-150LV) at a pressure of 0.8 to 1.0 atmospheres for 25 min. The *in vitro* regenerated plants developed in a growth chamber at +23 to 25°C, 40 μmol m⁻² s⁻¹ illumination, and a 16 h photoperiod.

Plants with a root system were transplanted into pots with a different ratio of substrate components: humus and sand, 80/20; peat and humus, 50/50; peat and sand, 80/20; peat, humus and sand, 50/40/10 and grown in the greenhouse.

Results

Molecular Assays and Population Analysis Since PCR is sensitive to several parameters, including magnesium, DNA structure, primer concentration, and annealing temperature during amplification, it is necessary to optimize reaction conditions and DNA detection systems to improve the efficiency of microsatellite analysis (Rahman *et al.* 2000).

Pairs of primers for each of the eight microsatellite SSR loci show single-locus drawings of all 28 *P. pruinosa* samples collected in Kazakhstan's Almaty and Zhetysu regions. The dimensions of the alleles observed in the 28 samples are shown in Table 3.

Based on the results of microsatellite analysis, the eight most effective markers were selected: ORPM344, PMGC2571, PMGC2679, WPMS5, WPMS14, WPMS20, PMGC2852, and PMGC2060. A total of 98 SSR amplified fragments were detected in the samples examined, with 16 of them being polymorphic. Among the 28 samples collected, 21 had unique microsatellite genotypes and 23 loci were polymorphic. The polymorphism level is 16%. A total of 23 loci were obtained, where the length of DNA fragments obtained ranged from 75 to 330 bp. All samples examined had an average of 2.88 amplicons per locus, with the highest being four loci (PMGC 2571, PMGC 2852, and ORPM 344) and the lowest being one locus (WPMS 20).

The samples were characterized using eight polymorphic primers from the SSR marker system. These eight primers gave a total of 98 strong and clear bands, with an average of 21.78 bands per primer in 28 samples. The average percentage of polymorphism was 12.21%, which indicates that it is present (Table 3.). The primers PMGC 2571 and WPMS 20 showed the highest (22) and lowest (6) number of correlated bands, respectively. The highest (1.984) and lowest (1.136) number of effective alleles (n_e) were observed for primers WPMS20 and PMGC2852, respectively, with a mean value of 1.700. The maximum (0.689) and minimum (0.211) Shannon information index (I) values were observed for primers WPMS20 and PMGC2852, respectively, while the mean Shannon information index was 0.558. The maximum gene diversity (h) (0.496) was recorded for the WPMS20 primer, while the lowest gene diversity (0.113) was observed for the PMGC2852 primers; the mean gene diversity was 0.387. The maximum genetic distance on the Nei scale was 1.3437

Table 3. The simple sequence repeats (SSRs) marker system determines to study *Populus pruinosa* Schrenk germplasm genetic diversity

Primer	Total bands	Polymorphic bands	Polymorphism, %	Ne*	h**	I***
ORPM 344	13	2	15.38	1.632	0.365	0.541
PMGC**** 2571	22	6	27.27	1.699	0.392	0.534
PMGC 2679	10	0	0	1.923	0.479	0.672
WPMS 5	11	0	0	1.766	0.430	0.620
WPMS 14	8	0	0	1.742	0.426	0.6172
WPMS 20	6	0	0	1.984	0.496	0.689
PMGC 2852	20	6	30	1.136	0.113	0.211
PMGC 2060	8	2	25	1.721	0.396	0.577
Mean	21.78	3.56	12.21	1.700	0.387	0.558
Total	98	16				

* N_e , effective number of alleles; ** h , Nei's gene diversity; *** I , Shannon's information index; *****PMGC* - Poplar Molecular Genetics Cooperative

between 2 samples from the Zhetysu region and 20 samples from the Almaty region, and the minimum was 0.0445.

The study samples had their genetic diversity and population structure assessed using clustering methods such as STRUCTURE and Popgene 1.32. Previous studies show that the model structure is more reliable and understandable than other approaches (Deacon *et al.* 2017). The results showed that the samples collected from the two areas were divided into four groups: group I, group II, group III, and the unclassified group. Group I contained 3 out of 28 (22, 21, 17), that is 10.17% of the total number of samples; group II contained 4 out of 28 (1, 4, 15, and 3), that is 14.29% of the total number of samples; group III contained 5 out of 28 samples (13, 14, 27, 8, and 9), that is 17.86%; and the fourth group was unclassified and consisted of 16 samples (2, 5, 6, 7, 10, 12, 16, 18, 19, 20, 23, 24, 25, 26, 28) and accounted for about 57% of the total number of samples. The population structure included all samples collected from *P. pruinosa*, which is an obvious result of the population structure when compared to other populations. The lack of clarity in loci in the structure of collected specimens leads to the isolation of unclassified populations, which may be caused by the mixing of genomic material (Fig. 2).

Results of the Microclonal Propagation Development Study The study of various methods of *in vitro* introduction showed that for the production of aseptic plants, the most effective use of single-tree cuttings 6 to 7 cm long with 1 to 2 buds and sterilization from saprophytic microflora with bleach NaOCl (1:1) 10 min and a solution of 0.1% HgCl₂ 5 min followed by three times sterile distilled water. The regeneration rate *in vitro* was 60 to 68%. Plants, as shown in Fig. 3, have active growth and development, and their leaves are bright green.

The method of *in vitro* inoculation by germination of shoots from lignified cuttings in laboratory conditions followed by planting on a nutrient medium was ineffective. Plants had poor growth and development, leaves were light green and vitrified, necrosis was observed on shoot tips,



Figure 3. Introduction of *P. pruinosa* into *in vitro* culture.

and callus was formed at the base of shoots. The number of aseptic plants obtained was 20 to 35%.

In addition to saprophytic microflora, plants can develop pathogenic microflora that are not killed by sterilization. If infected plants are planted on nutrient media, the pathogenic microflora will develop over time and may kill the plants. To avoid this, when culturing plants on a nutrient medium for clonal micropropagation, the basal part of shoots was isolated at the same time and placed on screening medium 523, which helps to detect pathogenic microflora within 3 to 5 d (Viss *et al.* 1991) (Fig. 4).

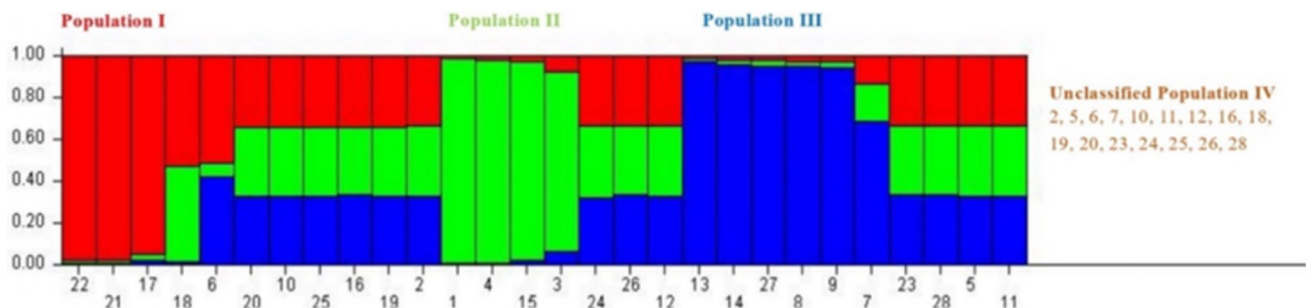


Figure 2. Population analysis through STRUCTURE software of 28 *Populus pruinosa* Schrenk samples using the simple sequence repeats (SSRs) marker system.

Figure 4. Verification of latent infection of explants on 523 medium: (a) absence of infection; (b) bacterial infection.

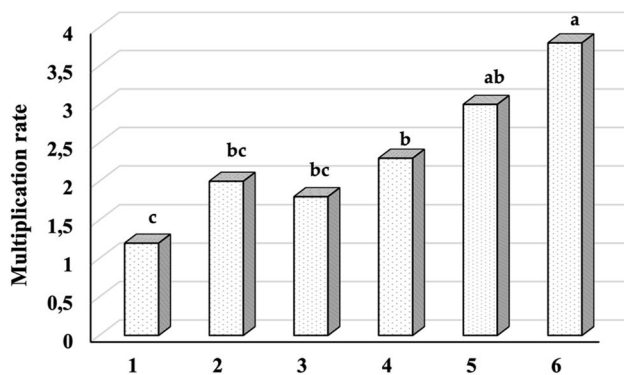
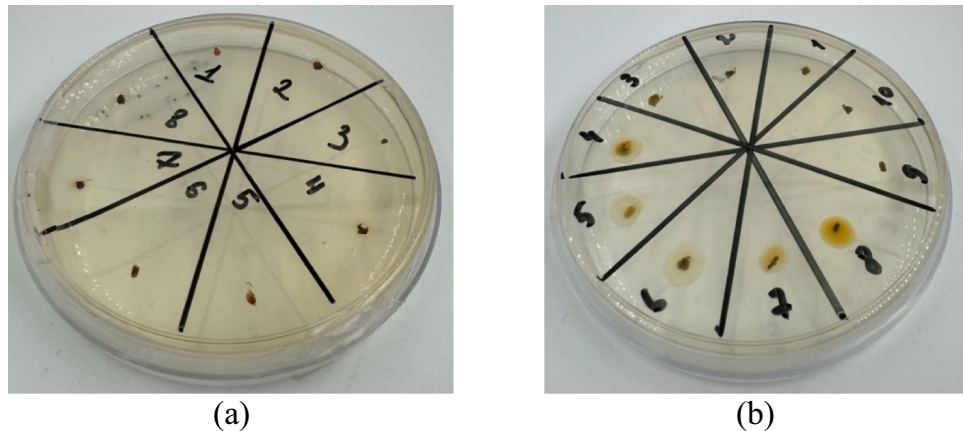


Figure 5. Effect of nutrient medium composition on clonal micropropagation of *Populus pruinosa* Schrenk (mean): (1) $\frac{1}{2}$ Murashige and Skoog (MS), 20.0 g L⁻¹ Glucose, pH 5.7; (2) MS, 0.1 mg L⁻¹ 6-benzylaminopurine (BAP), 0.02 mg L⁻¹ Gibberellic acid (GA), 30.0 g L⁻¹ Sucrose, pH 5.7; (3) Woody Plant Medium (WPM), 0.1 mg L⁻¹ BAP, 0.02 mg L⁻¹ GA, 20.0 g L⁻¹ Glucose, pH 5.7; (4) Driver and Kuniyuki Walnut (DKW) medium, 0.5 mg L⁻¹ Thiamin, 0.1 mg L⁻¹ BAP, 0.02 mg L⁻¹ GA, 20.0 g L⁻¹ Glucose, pH 5.7; (5) MS, 0.1 mg L⁻¹ BAP, 0.02 mg L⁻¹ GA, 20.0 g L⁻¹ Glucose, pH 5.7; (6) $\frac{1}{2}$ MS, 0.01 mg L⁻¹ BAP, 0.02 mg L⁻¹ GA, 20.0 g L⁻¹ Glucose, pH 5.7; a–c significant difference between the results obtained with different variants of experiments. 'ab' and 'bc' are experimental variants with no significant differences.

During different periods of cultivation, there have been instances where some shoots have shown signs of bacterial infection, including darkening of the base of the shoots and leaves, and growth media clouding, which eventually led to their death. Testing on screening medium 523 showed the presence of bacterial infection. The surface sterilization of the primary explants contributed to the elimination of fungal infections but was ineffective against bacterial infections. The infected plants were discarded.

The optimal composition for growth and micropropagation was established by transplanting small cuttings onto different nutrient media after leaf formation (Fig. 5).

The results of optimizing the nutrient medium for the growth and propagation of *P. pruinosa* indicated that using

sucrose did not result in a propagation rate above 2, and the plants were not pleased. The leaves and stems were light green, and 60% of the plants were vitrified. The use of glucose has led to an increase in plant multiplication rate. According to Fig. 6, the index reached an average of 3.8 to 4.5 on $\frac{1}{2}$ MS medium with 0.01 mg L⁻¹ BAP, 0.02 mg L⁻¹ GA, 20.0 g L⁻¹ glucose, pH 5.7. The average length of shoots in 4 to 6 wk was 5.4 to 6.0 cm, and the number of new shoots formed up to 4 to 6. The plant condition was unsatisfactory on nutrient medium WPM, regardless of glucose use, and the multiplication rate varied from 1.8 to 2.3. The average plant height during 41 d of cultivation was 1.1 cm. The leaves of most plants turned yellow and showed redness, along with calluses and strong vitrification. However, 44% of plants formed a root system. On DKW medium with glucose, the overall condition of the plants was good. The multiplication rate was between 2.3 and 2.9. Callus and root systems were not observed. The color of the leaves and stem was green.

The propagated plants were transferred to a root induction media which contained MS with 0.5 mg L⁻¹ IBA, and $\frac{1}{2}$ MS with 0.5 mg L⁻¹ IBA. After 2 wk of using medium $\frac{1}{2}$ MS with 0.5 mg L⁻¹ IBA, the plants began to create a root system. By the end of 4 to 5 wk, plants had a root system formation rate of 91.1% (Fig. 7).

Plants with well-developed roots were transferred to pots with varying ratios of substrate components (peat, humus, and sand) and grown in greenhouse conditions (Figs. 8 and 9). During 3 to 4 wk, the rooting rate of plants in pots with substrate peat, humus and sand, 50/40/10, respectively, ranged from 67 to 77.50%. The rooting rate in other pots was not as high as 40 to 55%.

Male and female annual shoots with 2 to 3 buds had their apical segments sterilized, inoculated in aseptic conditions, and propagated separately using microclonal techniques. Separately cloning male and female trees was planned to restore the demographic structure of sex imbalance in populations, also for the possibility of using clones of male

Figure 6. Growth and micro-propagation of *Populus pruinosa* Schrenk on $\frac{1}{2}$ Murashige and Skoog medium containing 0.1 mg L^{-1} 6-benzylaminopurine, 0.02 mg L^{-1} Gibberellic acid, 20.0 g L^{-1} glucose, pH 5.7.



individuals for the greening of settlements, creation of protective forest belts in farms, and for forest plantations along roads and railways.

Discussion

Estimating the population condition (Singh *et al.* 2013; Sharma *et al.* 2020) can be achieved by genetic confirmation of *P. pruinosa* species collected from natural stands using microsatellite SSR markers. During sample collection in the Zhetysu and Almaty regions, 27 samples had clear morphological signs of being from the same species, but only one was uncertain. It is known that *P. pruinosa* can sometimes grow in a displaced form with *P. diversifolia* in natural stands (Dimeyeva *et al.* 2023). In this regard, molecular PCR analysis was conducted based on eight microsatellite markers. According to the genotyping results, all 28 samples collected were from the same *P. pruinosa* species.

The amplification products consisted of a single band for all *P. pruinosa* samples. It was also found that the presence of the locus at the WPMS 20 marker was unique for six samples from the Zhetysu region (PP1, PP17, PP8, PP9, and PP22) and Almaty region (PP1). The length and size of amplified DNA fragments that were produced ranged from 75 to 330 bp, and it is also noticeable that some samples were smaller than others. For example, as well as in studies by Mashkina *et al.* (2016), where the polymorphism level of 21 poplar samples was 77.1%. It was observed that a single primer contained multiple amplifier bands, with the WPMS 14 marker bands ranging from 220 to 250 bp, which confirms the polymorphism of the samples. Our studies indicated that this was present in

various samples. The WPMS 14 primers PP1, PP20, PP1, and PP10 were 100 bp long, while PP12, PP13, PP16, and PP17 were detected at 110 bp.

The current study on clonal micropropagation has results that are somewhat different from those in the literature. The optimal medium for clonal propagation of both male and female individuals of *P. pruinosa* was $\frac{1}{2}$ MS, 0.1 mg L^{-1} BAP-0.1, 0.02 mg L^{-1} GA, 20.0 g L^{-1} glucose, pH 5.7. The main difference is the use of glucose in a nutrient medium as a carbohydrate feed. There are differences in the composition and concentration of certain phytohormones. So, for example, the studies of Jiang *et al.* (2015) developed a breeding environment for the hybrid *Populus euramericana* 'Neva'. The best results on regeneration of shoots and leaves were obtained on the medium $\frac{1}{2}$ MS containing 0.5 mg L^{-1} BA and 0.1 mg L^{-1} NAA. The root system was formed using $\frac{1}{2}$ MS medium with IBA at 1.0 mg L^{-1} . The survival of plants in the soil was more than 80%.

Salah Khattab (2011) analyzed the impact of the medium composition on the formation of shoots and clones of poplar and aspen *in vitro* culture using diverse phytohormones derived from nutritious MS and WPM. As a result, on WPM medium, 0.1 and 0.2 mg L^{-1} BAP for *Populus tremula* and *Populus tremula* $\times$ *Populus tremuloides*, the coefficient of shoot formation was on average 4, and for *Populus alba* the most effective was MS with 0.2 mg L^{-1} BAP. For root formation, the optimal medium for *Populus alba* was MS, 0.4 mg L^{-1} IBA, and for *Populus tremula* and *Populus tremula* $\times$ *Populus tremuloides* MS, 0.02 mg L^{-1} IAA, 0.1 mg L^{-1} NAA, respectively.

Populus tremula shoots were grown *in vitro* by Peterinel *et al.* (2009). To obtain aseptic plants, 2% sodium hypochlorite was the most effective. The wintering

Figure 7. *In vitro* root system development.



buds collected in late February and early March had the highest vitality. *In vitro* culture introduction was best carried out with $\frac{1}{2}$ MS medium, 20.0 μM ZR, and 1.0 μM IAA. For microclonal propagation, MS medium, 20.0 μM ZR, 1.0 μM IAA was optimal, and successful rooting was achieved using $\frac{1}{2}$ MS medium, 20.0 μM ZR, 1.0 μM IAA. Thus, 1804 viable *Populus tremula* seedlings were obtained under *in vitro* conditions within 6 mo.

In another study, dormant silver poplar buds were collected from the Czech Republic. The results showed that it is optimal to use a basic MS medium for microclonal propagation and to reduce the MS medium to $\frac{1}{4}$ for root system production (Máchová *et al.* 2018).

Pavlichenko Vasiliy and Marina Protopopova determined the optimal concentration of indol-3-butyric acid (IBA) for the micropropagation of *Populus × berolinensis* in their research. The *in vitro* collection of poplar plants was used by researchers. It has been shown that 0.15 mg L^{-1} IBA in MS nutrient medium is the most suitable for efficient micropropagation of *Populus × berolinensis* (Pavlichenko 2023).

The difference in the composition of the media for clonal micropropagation may be influenced by the different species of poplars studied.

The use of the basic nutrient medium MS with a selection of different phytohormones and their concentration

Figure 8. Influence of substrate composition on plant survival *Populus pruinosa* Schrenk (mean): (1) humus and sand, 80/20; (2) peat and humus, 50/50; (3) peat and sand, 80/20; (4) peat, humus and sand, 50/40/10.

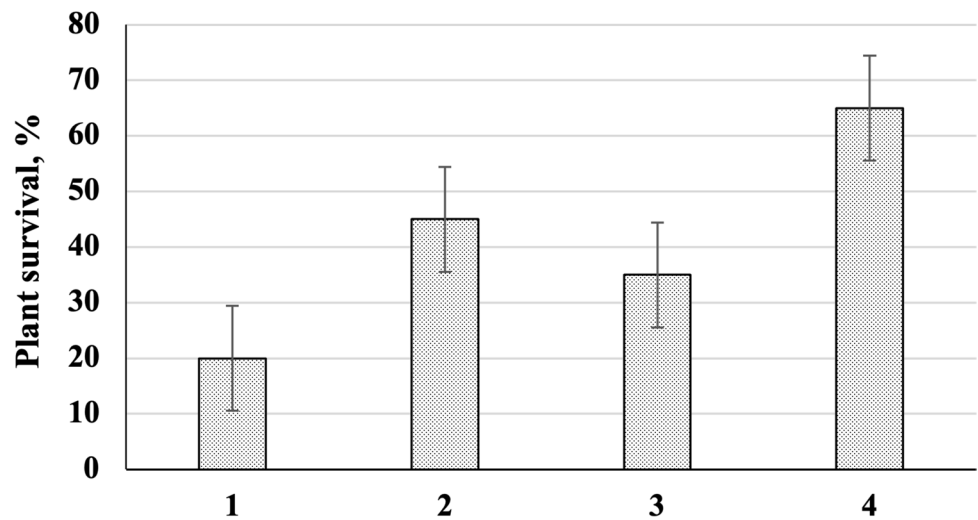


Figure 9. The growth and development of *Populus pruinosa* Schrenk within substrate pots.



allows successful inoculation *in vitro* culture, propagation, and obtaining root system.

Conclusions

All collected samples are of the same species, as confirmed by genotyping eight polymorphic simple sequence repeats (SSRs) markers. The results of the analysis of genetic diversity showed that the number of samples was divided into three populations, and the percentage of polymorphism was 12.21%.

Introduction of *P. pruinosa* into *in vitro* culture is effectively carried out in winter after completion of physiological dormancy from selected plus trees. For

introduction, it is effective to use lignified cuttings 6 to 7 cm long with 1 to 2 buds and sterilization from saprophytic microflora bleach NaOCl (1:1) 10 min and a solution of 0.1% HgCl₂ 5 min with washing three times with sterile distilled water.

Optimal medium for clonal propagation of both male and female individuals of *P. pruinosa* ½ Murashige and Skoog, 0.1 mg L⁻¹ 6-benzylaminopurine (BAP), 0.02 mg L⁻¹ Gibberellic acid (GA), 20.0 g L⁻¹ Glucose, pH 5.7, where the multiplication rate reaches an average of 3.8 to 4.5, and the length of shoots in 4 to 6 wk averages 5.4 to 7.0 cm, the number of newly formed shoots is 4 to 6.

The medium ½ MS with 0.5 mg L⁻¹ β-indolyl-3-butyric acid (IBA) is optimal for rhizogenesis of the studied turanga species.

The optimal substrate for transplanting plants from *in vitro* to pot culture is peat, humus and sand, 50/40/10, respectively.

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Data Availability The data presented in this study are available on request from the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

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