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RESEARCH PAPER

Peculiarities of *in vitro* cultivation of *Iris vorobievii* N.S. Pavlova (Iridaceae)

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ABSTRACT

Iris vorobievii is an herbaceous plant of the family Iridaceae, characterized by a small rhizome and low seed productivity. It occurs in the south of Khasan District in Primorye Territory, either singly or in small groups. The species is listed in the Red Data Book of the Russian Federation. As *I. vorobievii* is unstable under *ex situ* conditions, this paper addresses an alternative method for its preservation and propagation *in vitro* and examines some of its developmental features. A 1 % silver nitrate solution proved highly effective as a sterilizing agent. Concentrated sulfuric acid was found to have a positive effect not only on seed germination but also on the decontamination of mature seeds (up to 100 %). Germination rates under different sterilization schemes ranged from 46 % to 55 %. Cultivation on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L 6-benzylaminopurine promoted the formation of adventitious shoots and roots, whereas an increase in 6-BAP concentration had little effect on shoot multiplication. The application of naphthylacetic acid and thiadiazuron resulted in plant vitrification. Root system development occurred on MS without growth regulators. The main root of *I. vorobievii* was shown to die off during the first *in vitro* cultivation cycle. During adaptation to *ex vitro* conditions, the survival rate reached 70 %, and half of these plants completed their second and third growing cycles in the experimental greenhouse. Under *ex vitro* conditions, adventitious shoots developed in a few individual plants.

Keywords: 6-benzylaminopurine, microclonal propagation, rare species, endemic species

РЕЗЮМЕ

Бердасова К.С. *, Пьянова А.С., Миронова Л.Н. Особенности культивирования *in vitro Iris vorobievii* N.S. Pavlova (Iridaceae). *Iris vorobievii* – травянистое растение из семейства Iridaceae, имеющее небольшое корневище и обладающее низкой семенной продуктивностью. Произрастает на юге Хасанского района Приморского края отдельными особями или небольшими группами. Включен в Красную книгу Российский Федерации. В культуре *ex situ* нестабилен, поэтому в данной работе рассмотрен альтернативный метод сохранения и размножения данного вида *in vitro*, а также показаны некоторые особенности развития. Показана высокая эффективность использования в качестве стерилизующего агента 1 % раствора нитрата серебра. Отмечено положительное влияние концентрированной серной кислоты не только на прорастание, но и на обеззараживание зрелых семян (до 100 %). Всхожесть семян при разных схемах стерилизации составила от 46 % до 55 %. Культивирование на среде МС дополненной 0,5 мг/л 6-бензиламинопурина приводит к получению дополнительных побегов и образованию корней, увеличение концентрации 6-БАП незначительно влияете на мультипликацию побегов. Применение нафтилуксусной кислоты и тидиазурона приводит к витрификации растений. Образование корневой системы возможно на среде Мурасиге-Скуга без добавления регуляторов роста. Показано отмирание главного корня у *Iris vorobievii* на первом цикле культивирования *in vitro*. Выживаемость при адаптации к условиям *ex vitro* составила 70 %, половина из этих растений проходят второй и третий цикл вегетации в условиях экспериментальной теплицы. Дополнительные побеги в условиях *ex vitro* образуются у единичных экземпляров.

Ключевые слова: 6-бензиламинопурин, микроклональное размножение, редкий вид, эндемик

In the Far East, the genus *Iris* comprises 8–10 species (Alexeeva 2007, 2020). All species of the genus are perennial herbs varying in size, flower shape, and color, which makes them highly decorative. Moreover, some of them are valuable food and medicinal plants (Mironova 2022). *Iris vorobievii* N.S. Pavlova is an herbaceous annual or biennial plant, 20–35 cm tall. It has a thickened vertical rhizome about 1 cm long, with a dense cluster of adventitious roots (Alexeeva 2020, Mironova 2022). The species flowers in May, and the fruits ripen in July. In natural environments, it

reproduces vegetatively by forming side shoots. Seed propagation is hampered (Nikolaeva et al. 1985).

In Russia, *I. vorobievii* is found only in the south of Khasan District in Primorye Territory (Alexeeva & Mironova 2024). Up to ten locations are known, yet the species' range presumably extends to the border areas of China. It was first described from the surroundings of Kraskino (Primorye Territory). *Iris vorobievii* is a meadow-forest-steppe species, a xeromesophyte that occurs on open grassy slopes, river meadow terraces, and in sparse oak forests with *Quercus dentata* Thunb. It is distributed rather unevenly, either as individual plants or as separate groups comprising from a single to several dozen specimens in different locations. Most populations are located in the vicinity of settlements and are repeatedly subjected to anthropogenic or pyrogenic impact (Alexeeva & Mironova 2024). The species is listed in the Red Data Book of the Russian Federation (Geltman 2024).

Natural limiting factors include isolation and small population numbers, weak competitive ability of the species at the edge of its range, short life cycle (annual or biennial), and low seed propagation.

The first experiments on *I. vorobievii* cultivation *ex situ* took place at the Botanical Garden-Institute FEB RAS (Vladivostok) in the 1970s. As reported by L.N. Mironova, when maintained *ex situ*, the species is unstable; it flowers, bears fruit, and sets seeds. *Iris vorobievii* is also cultivated at the Komarov Botanical Institute of the Russian Academy of Sciences (St. Petersburg) under the supervision of N.B. Alexeeva, who, in turn, describes it as an unstable species. In St. Petersburg, it has not set seeds (Alexeeva 2009). Despite its beauty and ornamental value, *I. vorobievii* has not been involved in breeding (Alexeeva 2009, 2020).

The *Iris* species are of great interest to researchers in various fields. Thus, their morphological, molecular-genetic, and biotechnological features have been studied (Tikhomirova 2013, Liu et al. 2025, Xu et al. 2025). *In situ* development of *I. vorobievii* has also been examined, including some molecular and genetic aspects (Kozyrenko et al. 2009, Boltenkov & Artyukova 2023). It has also been studied *ex situ* (Stoletova & Mironova 2021, Alexeeva 2020). Still, exploring the development and propagation of this highly attractive species remains topical for conservation purposes.

Although protocols for the microclonal propagation of many *Iris* species (Ishmuratova 1999, Vechernina et al. 2004, Boltenkov & Zarembo 2005, Nabieva & Elisafenko 2012, Tikhomirova 2013, Taha et al. 2019, Tikhomirova 2021, Malaeva 2022, Pianova et al. 2025) have been developed, there is almost no data available for *I. vorobievii*. Therefore, its *in vitro* cultivation is the focus of this research. This method enables a significant increase in the multiplication factor by adding growth regulators to the nutrient medium, as well as monitoring plant development without external physical impact.

MATERIAL AND METHODS

The study was carried out in 2022–2025. The starting material for obtaining aseptic cultures was mature seeds from the collection of the Botanical Garden-Institute FEB RAS. In all experiments on the effect of growth regulators, microplants of the same age and size were used. The experiment was conducted in three replicates, with 20 plants per replicate.

Prior to scarification and sterilization, seed quality was assessed using a Stemi DV4 stereomicroscope (Carl Zeiss, Germany).

Seed scarification was carried out in two ways: soaking on filter paper in a refrigerator for 24 hours (3 Petri dishes with 13 seeds each) and treatment with concentrated sulfuric acid (soaking the seeds for 20 minutes, 2 samples of 20 seeds each). For sterilization, a mixture of a 1 % silver nitrate solution and 0.1 % Tween-80 solution (15 min) was used in the first case, and a 1% silver nitrate solution (15 min) in the second case. In both cases, the seeds were washed three times with sterile distilled water (Pianova et al. 2024).

Murashige–Skoog medium (Murashige & Skoog 1962) with 3 % sucrose and 0.7 % agar was used for cultivation. Before autoclaving, the pH of the medium was adjusted to 5.6–5.8. Autoclaving was carried out using a Sanyo MLS-3781L steam sterilizer (Japan) at 121°C for 20 min. Growth regulators were added to the medium after autoclaving. Sterilization of explants and passages were carried out in a BAVnp-01-Laminar-S-1.5 aseptic laminar flow cabinet (Russia).

Seeds were germinated on Murashige and Skoog nutrient medium without growth regulators (MS^0). Plants obtained from *in vitro* seedlings were placed on the surface of MS^0 medium and treated with growth regulators, such as 6-benzylaminopurine (6-BAP), naphthaleneacetic acid (NAA), indolyl-3-butyric acid (IBA), and thidiazuron (TDZ).

The culture room was maintained at a light intensity of 2 000–3 000 lux with white fluorescent light (Philips, Poland) and a photoperiod of 16 hours, while the room temperature was maintained at 23 ± 2°C.

A mixture of universal soil, peat, and vermiculite was used to acclimatize the clones to *ex vitro* conditions.

RESULTS AND DISCUSSION

In vitro cultivation methods include various experimental schemes, depending on the type of explant used and the purpose of the study. While some protocols involve the *in vitro* formation of organs such as shoots, roots, and seeds in order to regenerate complete plants, others imply the use of cell cultures and callus for various biochemical and physiological studies. However, the latter approach may lead to self-clonal variability of the studied samples; therefore, it is not considered in this work as an appropriate way of preserving the natural species.

The data presented in the literature on the germination of *Iris* seeds in the laboratory setting indicate the duration of the germination process (Belokurova et al. 2004, Soldatov & Karpova 2020) and the production of seedlings *in vitro*. We have developed two scarification methods and selected sterilizing solutions for each of them (Table 1).

Treating scarified seeds with silver nitrate solution and Tween-80 surfactant, followed by rinsing with sodium chloride solution, resulted in 84.6 % decontamination of the material. The second seed treatment scheme was more effective and yielded 100 % decontamination, which had

Table 1. Effectiveness of scarification and sterilization of *Iris vorobievii* seeds.

Treatment scheme	Sterilization effectiveness, %	Germination, %
Soaking in a cold chamber (6 ºC) → 1% $AgNO_3$ + 0.1% Tween-80 → 1% NaCl → 3-fold washing with sterile H_2O dist. → nutrient medium	84.6 ± 1.0	46.2 ± 1.3
H_2SO_4 conc. → 1% $AgNO_3$→ 1% NaCl → 3-fold washing with sterile H_2O dist. → nutrient medium	100.0 ± 0.0	55.0 ± 1.2

Note. Data are presented as persentages ± standard error (SE).

also been previously shown for another *Iris* species (Pianova et al. 2025). The use of concentrated sulfuric acid is highly effective because it not only disrupts the seed coat but also destroys fungal and bacterial spores, providing the first step in seed sterilization. This chemical agent is the most effective for obtaining *in vitro* cultures of *Iris* species. The scarification methods employed in this study enable germination on the 36th day, indicating a shorter time for obtaining seedlings of the rare *I. vorobievii*. In this case, 46 % to 55 % of the seeds germinate, which is quite high for a species with low seed productivity (Mironova & Kalinkina 2022). Storage of seeds of this species in a refrigerator (6 ± 1°C) reduces their germination rate to 11–16.7 %.

Plants obtained from the seeds were placed on nutrient media to induce multiplication and rhizogenesis (Table 2).

In studies on the microclonal propagation of other *Iris* species, a positive effect of 6-BAP on plant propagation has been noted (Nabieva & Elisafenko 2012, Pianova et al. 2025); therefore, it was used to induce multiplication in *I. vorobievii*. For rhizogenesis induction, IBA and NAA were added to the medium. In addition, thidiazuron was used.

Adding 6-BAP at a concentration of 0.5 mg/L led to the formation of roots and adventitious shoots, with a multiplication factor of 2. To obtain more adventitious shoots, the concentration of 6-BAP was increased to 1 mg/L. However, the multiplication factor rose only to 3, and the plants did not develop roots. Rhizogenesis in *I. vorobievii* was observed on MS^0 nutrient medium. The development of both shoots and roots at the lower concentration of 6-BAP suggests an optimal equilibrium between exogenous cytokinin and endogenous auxins within the explant tissue. Similar data have been reported for other rare irises. For instance, in a protocol for *I. sibirica*, low to moderate concentrations of cytokinins frequently allow initial organogenesis without completely suppressing root primordia initiation (Nabieva & Elisafenko 2026). At a concentration of 0.5 mg/L, 6-BAP likely provides sufficient proliferative activity of cells without substantially suppressing endogenous auxin signals, thereby creating conditions for root initiation. An increase in the dose to 1 mg/L, on the contrary, shifts the hormonal balance toward cytokinin dominance, which, as described in a number of studies, promotes shoot formation and callusogenesis while suppressing root differentiation.

During the micropropagation process, intense shoot proliferation was observed in one of the obtained microclones on a growth-regulator-free medium; these shoots subsequently rooted and continued to proliferate actively (Fig. 1). This phenomenon is highly atypical for the species under *in vitro* conditions. According to L.N. Miro-

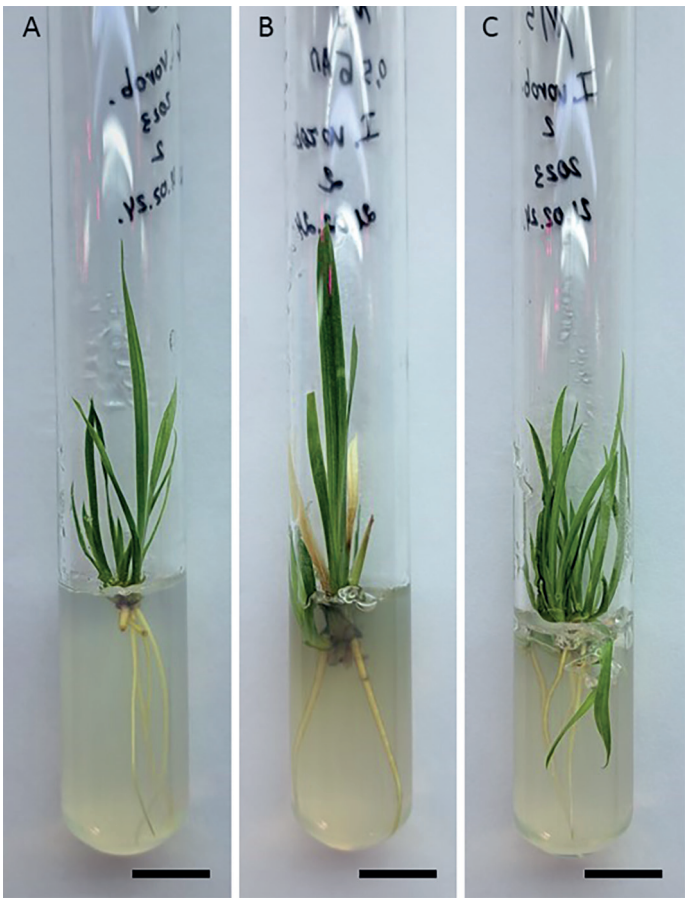

Table 2. Impact of growth regulators on *Iris vorobievii*

Expl. No	Growth regulator concentration, mg/l				Morphogenic response	Multiplication factor
	6-BAP	IBA	NAA	TDZ		
1	0.5	–	–	–	Roots, shoots	2.00 ± 0.24 [b]
2	1.0	–	–	–	Shoots	3.00 ± 0.22 [a]
3	–	0.5	–	–	Roots, shoots	2.00 ± 0.17 [b]
4	–	–	0.5	–	Vitrification, callus, shoots, roots in individual specimens.	2.00 ± 0.18 [b]
5	–	–	–	0.5	Vitrification, shoots, roots	3.00 ± 0.19 [a]

Note. Data are presented as the mean values ± standard error of the mean (SEM). Different letters within the same column indicate statistically significant differences between the experiments ($P \leq 0.05$, Tukey's test).

nova and L.M. Pshennikova, such specimens are extremely scarce in natural populations (Fig. 2B).

The use of 0.5 mg/L of IBA led to an increase in the multiplication factor, but the expected rhizogenesis process occurred in only 50 % of the plants. When 0.5 mg/L of NAA was added to the nutrient medium, root formation took place in individual specimens, while callus formation (in 7 %) or vitrification occurred in all plants cultured on this medium (Fig. 3A). This response can be attributed to the chemical nature of NAA. Unlike IBA, which is a naturally occurring compound that undergoes controlled enzymatic beta-oxidation, NAA is a highly stable synthetic auxin. It accumulates rapidly within plant tissues, often causing hyper-stimulation of cellular expansion. In irises and related monocots, high sensitivity to NAA frequently triggers massive callogenesis at the basal plate and alters chlorophyll and lignin accumulation, leading to the physiological water-logging characteristic of vitrification (Picoli et al. 2001, Polivanova & Bedarev 2022). Consequently, while NAA is an efficient regulator, it proves unsuitable for standard rhizogenesis protocols in *I. vorobievii*.

Adding thidiazuron to the medium resulted in the formation of adventitious shoots and rhizogenesis, but the plants exhibited vitrification and uncharacteristic leaf curling (Fig. 3B). This dual morphogenetic response can be attributed to the chemical nature of TDZ, which exhibits both cytokinin-like and auxin-like effects (Murthy et al. 1998, Ahmad & Shahzad 2018). Li and coauthors (2022) showed that TDZ induces a massive, rapid buildup of ethylene in the leaf blade relative to the petiole and abscission zone. In *in vitro* culture, this excessive gaseous ethylene accumulation frequently leads to cellular stress, abnormal leaf curling, and vitrification, which has also been recorded by us in *I. vorobievii*.

Similar effects of these growth regulators have not been observed when cultivating other plants and are not described in the literature for this genus. Following cultivation on media with TDZ and NAA, the resulting vitrified plants were transferred to media without growth regulators to assess the duration of their aftereffect. After three months of cultivation on MS^0 medium, the NAA effect was still present, while the effects of TDZ were decreasing. The ef-

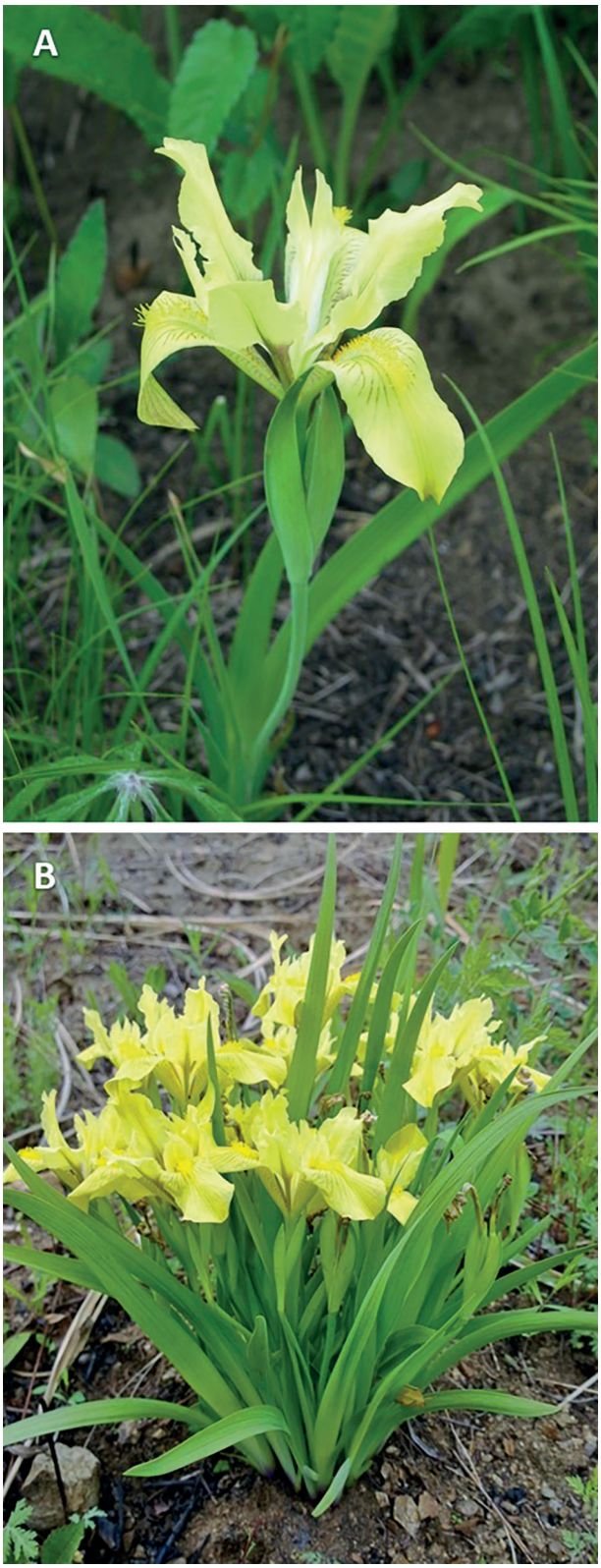

Figure 2 *Iris vorobievii* N.S. Pavlova plants in their natural habitat (Photo by L.M. Pshennikova)

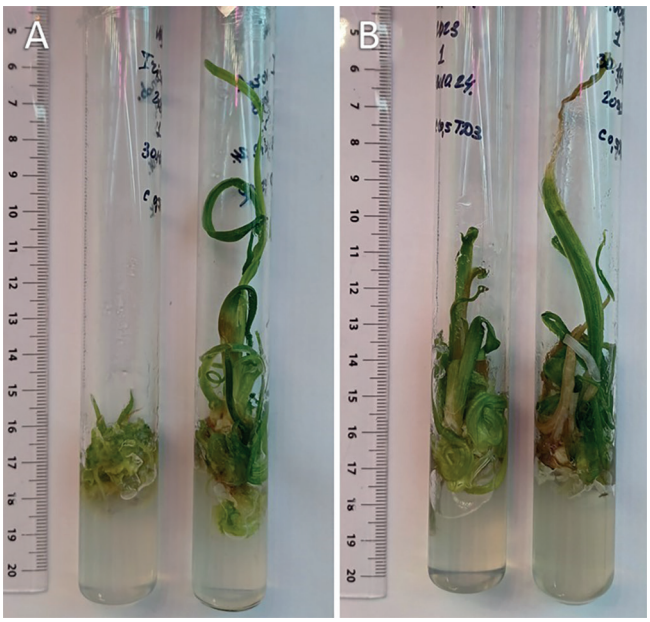

Figure 3 *Iris vorobievii* microclones during in *in vitro* cultivation: A – on MS+0.5 mg/l NAA, B – on MS+0.5 mg/l TDZ

fect of NAA decreased after six months of cultivation on medium without growth regulators. After one year of cultivation on MS^0, the growth regulators completely ceased to have an effect, and the plants developed normally, forming a root system and adventitious shoots.

Along with assessing the impact of growth regulators, we also observed the development of the root system *in vitro*. This study provides the first evidence that primary root formation and its death occur during the first cultivation cycle (Fig. 4), which corresponds to the first year of life *in situ*. Remarkably, proliferation of lateral shoots in the original plant occurs before the main root dies. Lateral roots in the mother and daughter plants develop independently of the death of the main root in the mother plant. N.B. Alexeeva (2020) reports that the rhizome of this species is vertical and does not form turfs. *In vitro* culture shows that after the main root dies, only lateral roots remain, and they are radially arranged relative to a small rhizome. This obviously limits the further growth of the rhizome, which, combined with low seed production, explains the narrow distribution of the species in nature.

Adaptation to *ex vitro* conditions was carried out in a mixture of peat, vermiculite, and universal soil in a 1:1:2 ratio. The survival rate during adaptation to soil was 70 %, and half of these plants completed their second and third growing cycles in the experimental greenhouse. Adventitious shoots were formed in single specimens under *ex vitro* conditions. Flowering was observed only in one sample during the third vegetation cycle, which corresponds to the third year of life *in situ*.

CONCLUSIONS

The study showed that a 1 % solution of silver nitrate is highly effective as a sterilizing agent (84.6–100 %). The positive effect of concentrated sulfuric acid has been noted not only on germination, but also on the decontamination of mature seeds. The germination rate of seeds under different sterilization schemes ranged from 46 % to 55 %. Cultivation on MS medium supplemented with 0.5 mg/L 6-BAP led to the formation of adventitious shoots and roots. The use of NAA and TDZ resulted in plant vitrification. Considering the obtained results, it is advisable to reduce the 6-BAP concentration in the medium to achieve the highest number of morphologically normal microclones for propagation and conservation. The main root of *I. vorobievii* was shown to die off during the first cultivation cycle. The survival rate during adaptation to *ex vitro* conditions amounted to 70 %.

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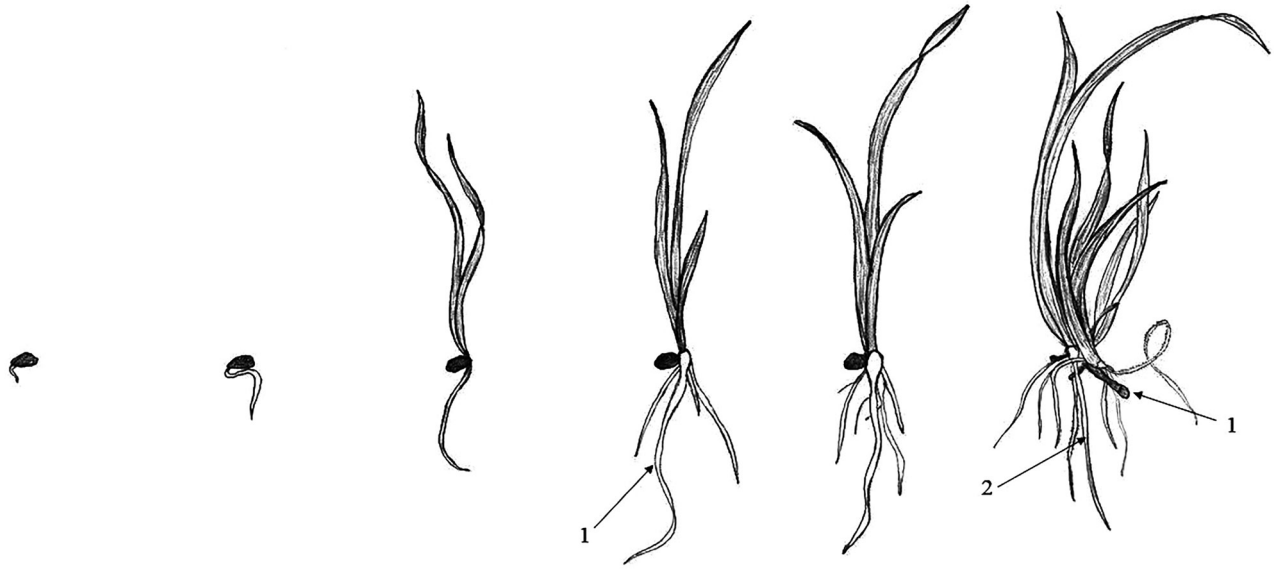

Figure 4 Ontogenesis of *Iris vorobievii in vitro*: 1 – main root of the mother plant, 2 – main root of the daughter plant (drawing by K.S. Berdasova)

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