Botanica Pacifica. A journal of plant science and conservation. 2025. 14(1):103–108
DOI: 10.17581/bp.2025.14113

RESEARCH PAPER

Optimization of seed scarification protocol and assessment of the effect of 6-BAP concentration on microplants of *Iris mandshurica* Maxim. (Iridaceae)

Anastasiya S. Pianova*, Kseniya S. Berdasova, Ludmila N. Mironova, Alexey V. Salokhin, Yuri E. Sabutski

Anastasiya S. Pianova *
e-mail: berdasova_as@mail.ru

Kseniya S. Berdasova
e-mail: k.berdasova@mail.ru

Ludmila N. Mironova
e-mail: lymironova@yandex.ru

Alexey V. Salokhin
e-mail: al-xv@mail.ru

Yuri E. Sabutski
e-mail: alixar2006@yandex.ru

Botanical Garden-institute FEB RAS,
Vladivostok, Russia

* corresponding author

Manuscript received: 18.01.2025
Review completed: 14.03.2025
Accepted for publication: 19.03.2025
Published online: 21.03.2025

ABSTRACT

The paper presents data on the methods of stimulation of *Iris mandshurica* seed germination and the effect of different 6-BAP concentration regimes on the multiplication and quality of microplants. The proposed scarification using H_2SO_4 made it possible to accelerate the germination of mature seeds many times, which occurred without the use of growth regulator. It was found that the formation of adventitious buds at the base of microshoots occurs at all concentrations of 6-BAP studied, however, the concentration of this phytohormone affects the quality of the resulting plant material. Addition of 1 mg/l 6-BAP to Murashige and Skoog basic medium increased the number of microplants as well as shoot height. Cultivation on Murashige and Skooga medium without regulators is sufficient to obtain a well-developed root system and does not require the use of additional growth stimulants. Adaptation of microclones to *ex vitro* conditions using commercial soil does not cause difficulties, and the plant survival rate is high and amounts to 76%.

Keywords: *in vitro* culture, micropropagation, growth regulators, 6-benzylaminopurine

РЕЗЮМЕ

Пьянова А.С., Бердасова К.С., Миронова Л.Н., Салохин А.В., Сабуцкий Ю.Е. Оптимизация протокола скарификации семян и оценка влияния концентрации 6-БАП на микрорастения *Iris mandshurica* Maxim. (Iridaceae). В статье представлены данные о способах стимуляции прорастания семян *Iris mandshurica* и влиянии различных режимов концентрации 6-БАП на мультипликацию и качество микрорастений. Предложенная скарификация с использованием H_2SO_4 позволила многократно ускорить прорастание зрелых семян, которое происходило без применения регулятора роста. Установлено, что образование адвентивных почек в основании микропобегов происходит при всех исследованных концентрациях 6-БАП, однако, концентрация этого фитогормона влияет на качество получаемого растительного материала. Добавление 1 мг/л 6-БАП к основной среде Мурасиге и Скуга позволило увеличить количество микрорастений, а также высоту побегов. Культивирование на среде Мурасиге и Скуга без регуляторов является достаточным для получения хорошо развитой корневой системы и не требует применения дополнительных стимуляторов роста. Адаптация микроклонов к условиям *ex vitro* при использовании коммерческого грунта не вызывает затруднений, при этом приживаемость растений высокая и составляет 76%.

Ключевые слова: культура *in vitro*, микроклональное размножение, качество, регуляторы роста, 6-бензиламинопурин

The genus *Iris* L. in the Russian Far East is represented, according to different authors, by 8–10 species (Alekseeva 2020). Some systematic issues of the genus remain still unresolved. The study of *Iris mandshurica* Maxim., *I. vorobievii* N.S. Pavlova and *I. humilis* Georgi of the section *Psammiris* (Spach) J.J Taylor. showed that all taxa have a rank of species (Alexeeva & Mironova 2007, Alexeeva 2018). Phylogenetic studies by Kozyrenko et al. (2009) also confirmed the species status of these taxa. The authors concluded that *I. mandshurica* and *I. vorobievii* are widespread in Primorye Territory, and *I. humilis* does not occur in this area. In a later work, Boltenkov & Artyukova (2023), based on chloroplast DNA sequences, revised the taxonomy of the *Psammiris* section, and the name *I. mandshurica* was assigned to the synonymy of *I. humilis*, although today this species is recognized as an independent species that is reflected in international databases: The International Plant Names Index, The World Flora Online, eFloras, The World Checklist of Vascular Plants. Both *Iris mandshurica* and *I. vorobievii*, these species are narrow endemics and were included in the Red Data Book of Primorye Territory and the Red Data Book of Russia (2024).

Iris mandshurica Maxim. is a perennial with a short creeping rhizome forming a loose turf. Flower stalks reach 20 cm in length, hollow inside, leaves are grayish-green, slightly sword-shaped, 18–20 cm long. Flowers are light yellow with bright yellow beard on the outer lobes of the perianth, with dark brown veining (Alekseeva 2020). This species is of interest as an ornamental and medicinal plant (Nikolaev et al. 2019).

Earlier works on *I. mandshurica* were aimed at studying the embryology, systematics, and structural features of the species (Wang & Zhuo 2006, Kozyrenko et al. 2009, Zhang 2011).

Despite the fact that for many *Iris* species there are currently protocols for microclonal propagation (Ishmuratova 1999, Boltenkov & Zarembo 2005, Nabieva 2012, Tikhomirova 2017, Malaeva 2022), for *I. mandshurica* data are practically not presented. In addition, most researchers use leaves, rhizomes

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or zygotic embryos isolated from seeds as explants (Nabieva 2010, Taha et al. 2019, Li et al. 2020, Tikhomirova 2021). Currently, there is insufficient information on microclonal propagation of *I. mandshurica* using seeds as a primary explant and further effective organogenesis. In databases we found only one article containing information on the introduction of *I. mandshurica* into *in vitro* culture (Belokurova et al. 2004). Our study focused on the development of a protocol with effective scarification of *I. mandshurica* seeds, their successful sterilization from immature and mature capsules. One of the objectives was to establish the most economically efficient concentration of the growth regulator 6-benzylaminopurine (6-BAP) to obtain the required volume of quality explants. This study is relevant for obtaining a large amount of plant material, for further study and for *in vitro* and *ex situ* conservation of the species, since the habitats of the studied species are vulnerable and are regularly subjected to anthropogenic pressures.

MATERIAL AND METHODS

The starting material for obtaining aseptic cultures was mature and immature seeds of *I. mandshurica* from the collection of the Botanical Garden-Institute FEB RAS.

Before scarification and sterilization of seeds, their quality was assessed using a Stemi DV4 stereomicroscope (Carl Zeiss, Germany), and additionally X-ray diagnostics was performed on a Faxitron MX-20 digital X-ray machine (USA). Fully formed seeds (Fig. 1A) with a normally developed embryo (Fig. 1B) were selected for the study.

Scarification, sterilization and sowing of seeds were carried out according to the following schemes (Fig. 2):

(a) seed washing in 0.1 % Tween-80 solution for 20 min., then three times rinsing with distilled water; sterilization in 1 % silver nitrate ($AgNO_3$) solution with 0.1 % Tween-80 for 25 minutes; the seeds were then treated with sterile 1 % sodium chloride solution, washed three times with sterile distilled water and planted on nutrient medium according to Murashige & Skoog (1962) (MS) with the addition of 0.5 mg/L 6-benzylaminopurine (MS+0.5 6-BAP);

b) pretreatment of seeds at full maturity stage with concentrated sulfuric acid for 20 minutes; sterilization with 1 % silver nitrate (15 min.) followed by treatment with 1 % sodium chloride solution; after sterilization, seeds were washed three times with sterile distilled water and placed on MS nutrient medium without growth regulators (MS^0);

c) the closed immature capsule was dipped in 96 % ethyl alcohol and ignited in a torch flame; then, seeds were extracted (at waxy stage) and placed on MS+0.5 6-BAP nutrient medium.

Mature (Control 1) and immature (Control 2) seeds sown on MS^0 medium served as controls.

To study the effect of various concentrations of 6-BAP, seven-day-old germs were placed on modified MS nutrient medium with different concentrations of growth regulator: 0.5, 1.0, 1.5, and 2 mg/ml. Induction of rhizogenesis was carried out on MS^0 and MS with the addition of indolyl-3-butyric acid (IBA). Vessels with plants were placed in a culture room with a constant temperature of 24 ± 2°C and a photoperiod of 16/8 h (light/dark). Illumination of racks with vessels was provided by cool-white fluorescent lamps (Philips, Poland). After 20 days of cultivation on MS media with growth regulators, morphometric indices of microplants and shoot formation efficiency were evaluated as the ratio of the number of shoots after 20 days to the initial number of shoots in each culture vessel. All experiments were conducted under the same physical conditions in three repetitions; hormone-free MS medium was used as a control medium. Data were entered into Microsoft Office Excel (2010) spreadsheet, where statistical processing and analysis of the results were performed. Data are presented as mean values and standard errors (P = 0.05). Clonally cultivated plants for adaptation to *ex vitro* conditions were transferred to nutrient peat soil of the company "Piter Pit" (Russia) containing nitrogen, phosphorus and potassium. Watering was performed with room temperature water as the soil dried up.

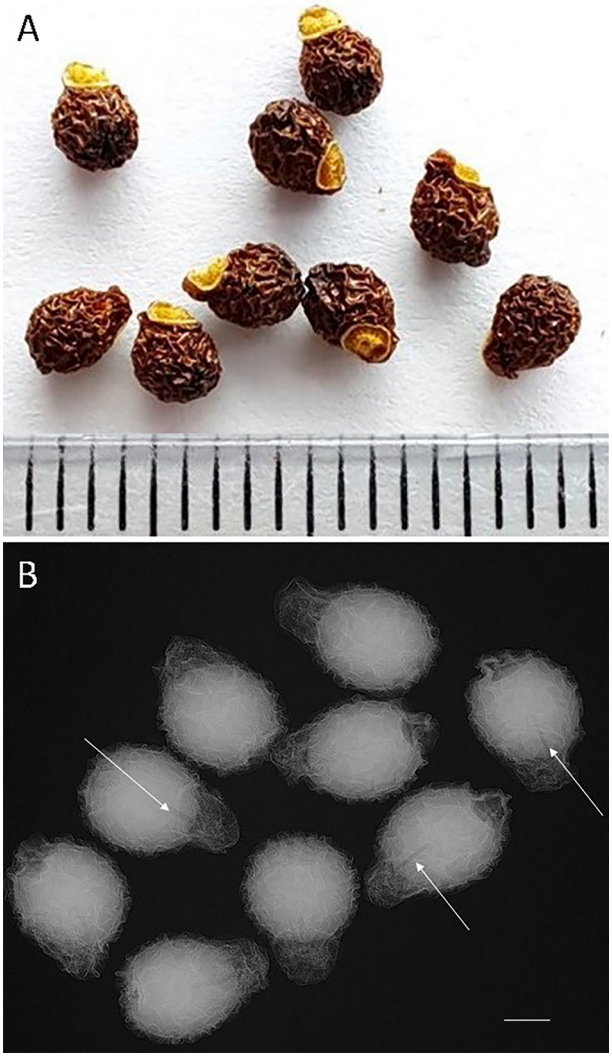

Figure 1 *Iris mandshurica* Maxim. seeds: A – seeds at full maturity stage; B – X-ray image, arrow indicates embryo. Scale bar: 1 mm

RESULTS

High seed sterilization efficiency (above 70 %) was observed in all experiments, and sterilization efficiency reached 100 % when concentrated sulfuric acid was used and immature capsules were burned (Table 1).

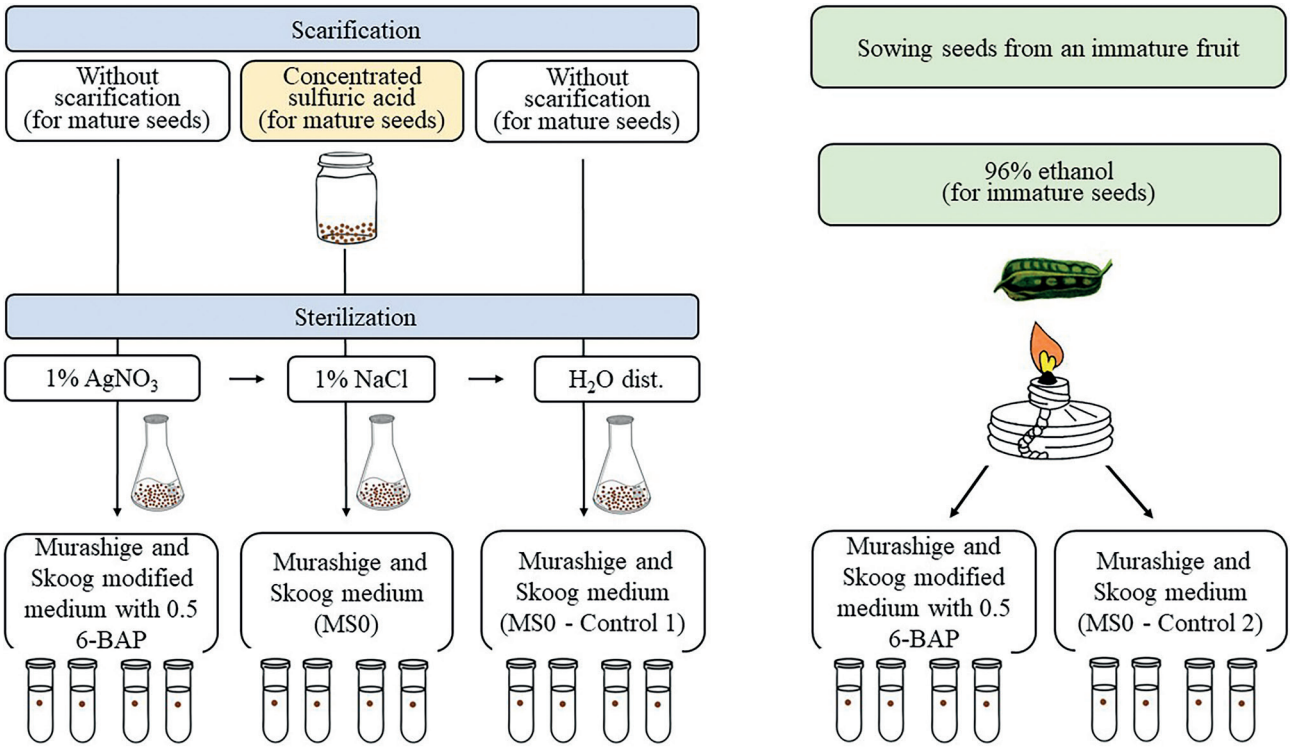

Figure 2 Scheme of scarification and sterilization of *Iris mandshurica* Maxim. seeds

All seedlings remained viable during further cultivation on the same media. Addition of 0.5 mg/L 6-BAP to the nutrient medium induced mass germination on 33rd day (both for seeds at full ripeness and seeds at waxy ripeness stage). The germination rate of mature seeds on this medium was 50 %, while that of immature seeds was 89 %. The use of concentrated sulfuric acid induced germination of seeds already on the 13th day of cultivation, germination in this method reached 80%. In control 1 (for mature seeds) mass germination was observed only on 43rd day, the percentage of germination was the lowest – 30 %. In control 2 (for waxy seeds), mass germination was recorded at 35th day and germination reached 72 % (Table 1).

When evaluating the effect of different concentrations of 6-BAP on the multiplication of *I. mandshurica*, the following relationship was observed: the number of newly formed shoots (multiplication factor) increased in proportion to the increase in the concentration of growth regulator in the medium (Fig. 3).

The highest value (6) was observed on MS medium supplemented with 2 mg/L 6-BAP. The average height of microplants at this concentration was 9.9 cm. Decreasing the concentration of 6-BAP to 1.5 mg/L resulted in a decrease of the coefficient to 5.8. However, the average height decreased significantly (4.6 cm). When 1 mg/L 6-BAP was used, the size of microplants reached maximum values, with an average height of 11.5 cm. Cultivation of *I. mandshurica* on MS with 0.5 mg/L 6-BAP resulted in the development of the lowest plants, with a multiplication factor marginally higher than the control. Subsequent subcultivations on media with high concentrations of the applied growth regulator resulted in a decrease in the size of microplants, affecting the quality of the plant material obtained.

Rhizogenesis was observed on nutrient medium MS^0. More efficient root growth was recorded when cultivated on nutrient medium MS+2 mg/l IBA. Cultivation on both media resulted in the formation of a root system able to positively transfer adaptation to *ex vitro* conditions. The only difference was the rate of root system growth. Adaptation of microclones using commercial soil of the company "Piter Pit" (Russia) was successful, the survival rate of plants was 76 %.

Table 1. Key indicators in the introduction of *Iris mandshurica* to *in vitro* culture: sterilization effectiveness, germination rate and germinating capacity.

Method of sterilization and germination	Sterilization efficiency, %	Germination rate, day	Germination, %
Sterilization by $AgNO_3$ and sowing of mature seeds on MS with 0.5 mg/l 6-BAP	71,4	33	50
Sterilization by $AgNO_3$ and sowing of mature seeds on MS^0 with pretreatment of H_2SO_4	100	13	80
Sterilization by $AgNO_3$ and sowing of mature seeds on MS^0 without pretreatment (Control 1)	71,4	43	30
Sterilization of immature pods by firing and sowing seeds on MS with 0.5 mg/l 6-BAP	100	33	89
Sterilization of immature pods by firing and sowing seeds on MS^0 (Control 2)	100	35	72

Note: H_2SO_4 concentrated. Processing time 25 minutes

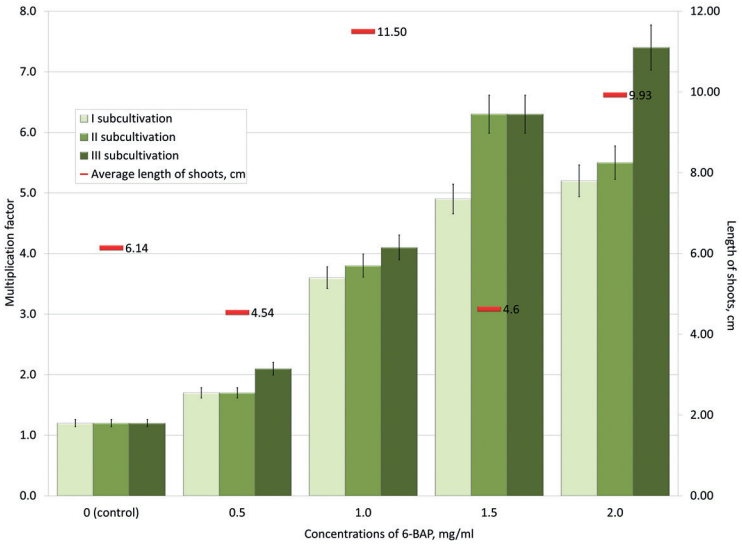

Figure 3 Effect of 6-BAP concentration on the *Iris mandshurica* multiplication factor and plants height

DISCUSSION

Seed germination is an important and complex process from a biochemical and physiological point of view. The *Iris* seeds with a long dormancy period cause difficulties in reproduction, including *in vitro* cultivation. Selection of adequate conditions of seed pretreatment and sterilization is important for germination improvement. Seed scarification is necessary in the study of seed productivity of representatives of the genus *Iris*. Thus, Soldatov & Karpova (2020) germinated seeds in Petri dishes before introduction into *in vitro* culture using three different methods (temperature and light fluctuation, freezing, seed coat disruption). Belokurova et al. (2004) scarified seeds of *I. mandshurica* for long periods of time (2 months of cultivation at 4°C). In our study, the possibility of significant acceleration of the scarification process of mature seeds was shown using sulfuric acid, which increases germination up to 80 % within 13 days (Table 1). High efficiency of sterilization in this case is due to the aggressive component, since concentrated sulfuric acid not only breaks the seed coat, but also destroys fungal and bacterial spores, providing the first step of seed sterilization. Thus, this chemical agent is the most effective in obtaining *in vitro* culture of *I. mandshurica*. However, sterilization of mature seeds with 1 % silver nitrate solution without treatment with sulfuric acid can also be used. This method has the advantage of a rather high degree of sterilization (more than 70 %), mass germination of seeds is registered only on 43rd day of cultivation. But the use of growth regulator in the nutrient medium during seed germination reduces the germination period by 10 days. Sowing mature seeds of *I. mandshurica* on nutrient medium containing 6-BAP is not an effective enough method for obtaining a large number of seedlings, because in this method the germination rate is only 50 %, which is almost 30 % less than when using sulfuric acid and cultivation on nutrient medium MS^0 (Table 1). In addition, the use of growth regulators in seed germination increases the cost of seedling production. Nevertheless, this method can be used in rare species conservation studies as an alternative to traditional methods.

Since one of the most commonly used growth regulators is cytokinin 6-benzylaminopurine, we also used it for induction of multiple shoot formation. The concentration of this growth regulator was found to affect the quality of the plant material obtained. Application of 0.5 mg/L 6-BAP did not lead to the expected results. We believe that to obtain a large amount of plant material of *I. mandshurica*, the use of this concentration did not lead to the expected results. Since there is no significant difference relative to the control (MS^0). The optimal concentration for obtaining a large number of quality clones of *I. mandshurica* is 1 mg/l of 6-BAP medium. It was noted that using this concentration, the multiplication factor does not decrease with repeated subcultivation, and the quality of plants remains high.

It is worth noting that root formation was observed when daughter plants were cultured on MS^0 medium (Fig. 2).

Addition of the growth regulator indolyl-3-butyric acid to the nutrient medium resulted in faster growth of the root system with the formation of additional shoots. It becomes obvious to use IBA when it is necessary to accelerate rooting processes in *I. mandshurica* for large-scale production of seedlings. For basic

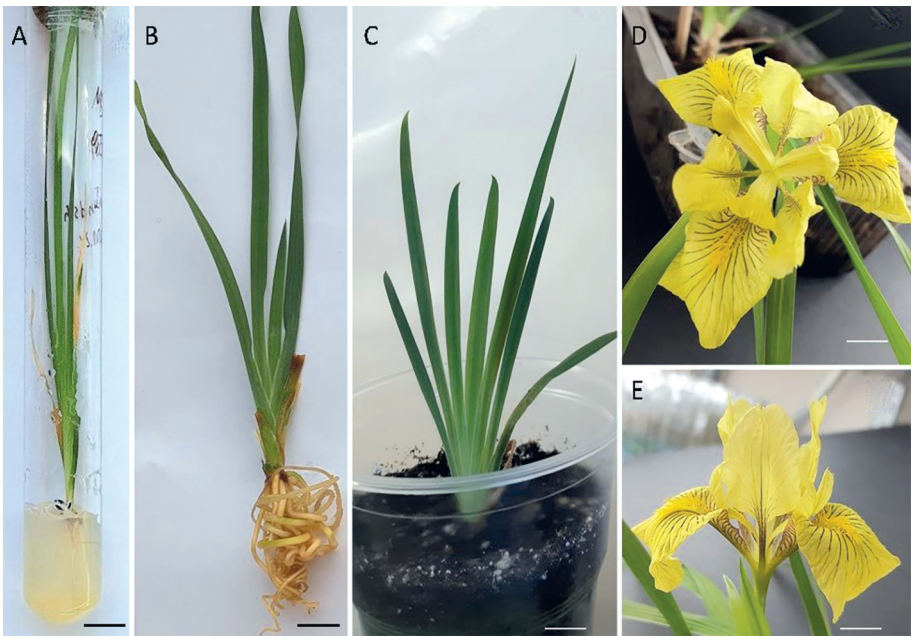

Figure 4 *Iris mandshurica* plant cultivated *in vitro* in growing medium MS + 1 mg/l of 6-BAP (A) and transferred to MS0 for growth of a root system (B). Adaptation to *ex vitro* conditions (C) and *ex vitro* flowering (D, E). Ruler interval: 1 cm

research it would be reasonable to use nutrient medium free of growth regulators. The difference was noted only in the rate of root system growth. The use of both media resulted in the formation of a root system capable of positively tolerating acclimatization to *ex vitro* conditions. Plants of *I. mandshurica* obtained by cultivation on MS+1 mg/L 6-BAP successfully adapted to *ex vitro* conditions and were resistant to temperature differences under experimental conditions (Fig. 4).

Such plants can be recommended for use in exposition plots of botanical gardens and in urban landscaping. Ying et al. (2016) also proposed the idea of the possibility of using *I. mandshurica* in landscape design due to its high ecological adaptability. In addition, *I. mandshurica* has medicinal properties (Nikolaev et al. 2019). Cultivation of microplants on nutrient medium with high concentrations of the applied growth regulator (MC+1 and MC+2 mg/L 6-BAP) had a positive effect on biomass growth. Therefore, it seems reasonable to use high concentrations of 6-BAP for the growth of medicinal raw materials.

CONCLUSION

Selection of adequate conditions for scarification of *I. mandshurica* seeds can significantly increase the number of obtained seedlings. An important factor affecting the frequency of shoot formation in *I. mandshurica* during microclonal propagation is the concentration of 6-BAP in the nutrient medium. It is necessary to carefully control the concentration of 6-BAP when choosing the direction of work, as well as obtaining quality plant material. The use of IBA to accelerate processes of *I. mandshurica* rooting in the large production of planting material is advisory rather than mandatory. For the induction of rhizogenesis and root system growth, it is sufficient to use a nutrient medium free of growth regulators. These data should be taken into account when introducing *in vitro* culture of *I. mandshurica* from wild populations, as well as other representatives of the genus *Iris*. Successful adaptation of microclones to *ex vitro* conditions can be carried out when using commercial soil of the firm "Piter Pit" (Russia). In our case, the survival rate of plants when transferred to *ex vitro* conditions was 76 %.

ACKNOWLEDGEMENTS

The article was prepared within the framework of the state assignment of the Ministry of Science and Higher Education of the Russian Federation "Introduction, study and conservation of genetic resources of East Asia economically valuable plants" (Registration number: 122040800086-1). The research was done on the basis of unique scientific installation "*In vitro* living plant collection of the Botanical Garden-Institute FEB RAS".

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