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In vitro propagation of rare species *Rhodiola rosea* from Altai Mountains

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Keywords: *ex vitro* acclimatization, *in vitro* propagation, planting material, *Rhodiola rosea*.

Summary. The techniques of biotechnology are considered promising and highly-demanded for propagation and conservation of rare species of medicinal plants. The variety of natural forms of *Rhodiola rosea* makes it difficult to develop a single efficient *in vitro* protocol for this valuable and rare medicinal plant. The optimization of *in vitro* methods for specific populations to effectively mass production of planting material *R. rosea* is required. The paper provides the technique for *in vitro* propagation of *R. rosea* from the population of Altai Mountains, including sterilization of seeds in an aqueous solution of sodium hypochlorite, planting of seedlings on Murashige and Skoog medium (MS) supplemented with 5 μM 6-benzylaminopurine (BAP) and 2.5 μM α-naphthylacetic acid (NAA), rooting of microshoots on hormone-free ½ MS medium and acclimatization in the substrate consisting of peat and perlite at a ratio of 2 : 1. We have shown that no additional pre-sowing treatment is required for effective *in vitro* germination of *R. rosea* seeds. The seedlings of *R. rosea* exhibit a high germination rate and are a promising explant type for *in vitro* mass propagation of the plant. This technique provides a high yield of *in vitro* cultivated plants (6.3 plants per explant, 95–100 % rooting) and a high percentage of plant acclimatization (78 %). The average weight of roots (wet weight) during acclimatization and growth (7 months) was 12.2 ± 2.4 g. The developed technique of *in vitro* propagation of *R. rosea* allows both to conserve the natural populations of this rare plant and to obtain planting material for commercial cultivation.

Размножение в культуре *in vitro* редкого вида *Rhodiola rosea* с Алтая

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Ключевые слова: адаптация к условиям *ex vitro*, размножение *in vitro*, посадочный материал, *Rhodiola rosea*.

Аннотация. В настоящее время применение методов биотехнологии для размножения и сохранения редких видов лекарственных растений является перспективным и востребованным направлением. Разнообразие природных форм *Rhodiola rosea* (родиолы розовой) затрудняет разработку единого эффективного протокола размножения *in vitro* этого ценного и редкого лекарственного растения. Для эффективного использования

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технологий *in vitro* с целью массового размножения родиолы розовой требуется оптимизация методик для конкретных популяций. В результате работы предложен способ размножения *in vitro R. rosea* из популяции Горного Алтая, включающий стерилизацию семян в водном растворе гипохлорита натрия, высаживание проростков на питательную среду Мурасиге и Скуга (MS), дополненную 5 μM 6-бензиламинопурина (БАП) и 2,5 μM α-нафтилуксусной кислоты (НУК), укоренение микропобегов на безгормональной среде ½ MS и адаптацию в субстрате состоящем из торфа и перлита в соотношении 2 : 1. Нами показано, что в культуре *in vitro* для эффективного прорастания семян родиолы розовой не требуется дополнительная предпосевная обработка. А проростки родиолы розовой характеризуются быстрыми темпами роста и являются перспективным типом экспланта для массового размножения этого растения в культуре *in vitro*. Данная технология обеспечивает высокий выход растений-регенерантов в культуре *in vitro* (6,3 растений на эксплант; 95–100 % укоренение) и 78%-ю адаптацию растений к условиям *ex vitro*. Средняя масса корней (сырой вес) за период адаптации и доращивания (7 месяцев) составила 12,2 ± 2,4 г. Размножение в культуре *in vitro* родиолы розовой позволяет, как сохранять природные популяции этого редкого растения, так и получать посадочный материал для коммерческого выращивания.

Introduction

Rhodiola rosea L. (golden root) from the family Crossulariaceae is a medicinal plant with a wide range of secondary metabolites, the most important of which are salidroside and alcohol derivatives of cinnamic acid and glycosides (phenylpropanoids) – rosavine, rosin and rosarin (which are referred to as rosavins) accumulated in the plant rhizome. There are about 58 species of the genus *Rhodiola* (Ohba, 2005), but rosavins are found only in *R. rosea*. This species is of great value both in traditional medicine and in natural food industry due to its unique composition (Kurkin, Zapesochnaya, 1986; Saratikov, Krasnov, 2004; Panossian et al., 2010; Stepanova et al., 2016). Nevertheless, global commercial demand for *R. rosea* is almost exclusively satisfied by wild harvested plants. *R. rosea* is very popular among the local people, which leads to uncontrolled harvesting of this plant and, hence, to depletion of natural wild populations. Currently, *R. rosea* is listed as endangered species and included in the Red Data Book of the Russian Federation (Nekratova, 2008). The species is recommended for inclusion in the IUCN Red List of Threatened Species.

Thus, the conservation of the genetic diversity of natural populations of *R. rosea* and provision of the pharmaceutical industry with this kind of plant material is possible only through commercial cultivation (Galambosi, 2005). It is known that *R. rosea* can be vegetatively propagated through cuttings of the underground part of the plant. However, this process is very laborious and slow to be used in large-scale commercial cultivation. The main problems of cultivation of plants from seeds are low germination rate and mass death of seedlings in the open ground, which requires greenhouses to grow immature plants. This process takes two years, after which the plants can be transplanted to the open ground. A very promising technique for mass production of the planting material is to employ clonal micropropagation.

The first studies on the *in vitro* cultivation of *R. rosea* were carried out 30 years ago (Kaftanat et al., 1988). A number of papers are devoted to the study of the micropropagation characteristics of this species. They consider the issues related to the effect of the medium composition and of various types of explants on the organogenesis and regeneration of *R. rosea* cultivated *in vitro* and identification of factors inducing these processes (Furmanowa, 1995; Ishmuratova, 1998; Yin et al., 2004; Tasheva, Kosturkova, 2010; Bae et al., 2012). A significant number of studies reported on the cell culture and genetically transformed roots of *R. rosea* as producers of biologically active substances, and on the techniques to enhance their biosynthesis (Furmanova et al., 2002; György, 2006; Krajewska-Patan et al., 2007; Grech-Baran et al., 2015; Marchev et al., 2016; Lütken et al., 2017). However, only a few papers address the production of the planting material for commercial cultivation (Platikanov, Evstatieva, 2008; *Rhodiola rosea*…, 2015). Considering that the species *R. rosea* is characterized by a high intraspecific morphological and biochemical variability, it is necessary to optimize the *in vitro* method for specific populations of this species (Ishmuratova, 1998; Kim, 1999; Zakhozhyy, 2006; Poletaeva et al., 2013).

The paper aims to develop the biotechnology technique for obtaining *R. rosea* planting material. The resulting method has the potential to be used for reintroducing *R. rosea* to mountain areas and for commercial cultivation of this species.

Materials and methods

Rhodiola rosea L. is a rare, arctic-alpine species with an almost circumpolar distribution. This

herbaceous perennial is widespread at high altitudes in the arctic and mountainous regions of Europe, Asia and North America (Nekratova, 2008). The most promising plants for introduction into culture with respect to the content of secondary metabolites, in particular salidroside, are *R. rosea* plants from the Republic of Altai. Introductory experiments performed earlier showed the ability of *R. rosea* to accumulate salidroside (up to 1.6 %) and to exhibit high biological productivity, which makes it a good candidate for cultivation in different soil climatic zones, in various altitude belts and on the plain (Poletaeva et al., 2013).

The object of the study was *R. rosea* plants from the Republic of Altai growing on the southern slopes of the Iolgo range, Karakol lakes, 1800–2000 m a. s. l.

Rhodiola rosea was cultivated *in vitro* at the Biotechnology Laboratory, Central Siberian Botanical Garden SB RAS (Novosibirsk). Sterilization was fulfilled in a laminar flow hood (Lamsystems, Russia). *R. rosea* seeds were the material for *in vitro* cultivation, development and optimization of the micropropagation technology. Surface sterilization was performed in accordance with the following scheme: seeds were immersed in 70 % ethanol for 30 seconds and then in 20 % Domestos (sodium hypochlorite) solution for 20 minutes (orbital shaker, 100 rpm) followed by three-time washing with sterile distilled water. For *in vitro* seed germination, a 0.6 % aqueous solution of agar (Difco, USA) was used.

Most of the studies on *R. rosea* have shown that Murashige and Skoog medium (MS) (Murashige, Skoog, 1962) was most optimal for propagation of this species, and therefore it was taken as a basic medium. During shoot multiplication, the medium was supplemented with 10 μM BAP (6-benzylaminopurine), zeatin and TDZ (thidiazuron) at a concentration of 1 : 5, and with 5 μM BAP in combination with 2.5 μM NAA (α-naphthylacetic acid). For rooting, half-strength MS medium (½ MS) supplemented with 5 μM IBA (indole-3-butyric acid) was used. The source of carbohydrates was sucrose (30 g/l), and the medium pH was adjusted to 5.8 before autoclaving. The passage lasted 30–35 days.

The conditions of explant cultivation were as follows: photoperiod – 16/8-hour light/dark cycle, illumination intensity – 2–3 klx, and temperature – 24 ± 1 °C. Seeds were germinated in the light and in the dark.

To conduct the *in vitro* study of the morphogenesis, the following parameters were considered: propagation coefficient as the number of developed shoots in one explant (pieces/explant), rooting (%), and the number of roots per explant (pieces/explant).

The *in vitro* cultivated plants were acclimatized to non-sterile conditions in cassettes, the substrate consisted of perlite and peat (1 : 2). The substrate was wetted with fungicide solution (Bactofit, Russia), at a concentration of 10 g per 5 liters of water or a weak solution of potassium permanganate to prevent and control fungal and bacterial plant diseases. Within the first five days, the plants were covered with a transparent film to provide high humidity for acclimatization. The plants were grown indoors at 24 ± 2 °C, illumination intensity of 2–3 klx within a photoperiod of 16/8-hour light/dark cycle for 30 days. After a month, the plants were transplanted into 10 × 10 × 11 cm containers by transferring to a soil mixture that consisted of peat and sand at a ratio of 2 : 1 and were grown for 7 months under greenhouse conditions.

Statistical processing of the results and analysis of the obtained data were carried out using Microsoft Excel 7.0. All the experiments were performed in 2 replicates of 20–30 plants in each replication. The data are presented as mean values and confidence intervals ($p \leq 0.05$)

$X \pm t \times S_x$, where X is sample mean t is Student's test,

S_x is sampling error.

Results and Discussion

Significant progress has been made in the field of biotechnology of medicinal plants – cultivation of cells, tissues and organs of *Panax ginseng*, *Catharanthus roseus*, *Dioscorea deltoidea* and species of the genus *Digitalis*, *Arnica montana*, *Rauwolfia* and *Galanthus* (Ramachandra, Ravishankar, 2002; Verpoorte et al., 2002; Khan, 2009). A number of papers address the micropropagation of *R. rosea* (Furmanowa, 1995; Ishmuratova, 1998; Yin et al., 2004; Tasheva, Kosturkova, 2010; Bae et al., 2012), however, the results of these studies are contradictory due to large polymorphism of this species and the presence of numerous ecotypes.

In vitro cultivation

We have shown that *R. rosea* seeds germinate after 2–3 days of cultivation in both light and dark cycles (Fig. 1). The seed germination of the Mountain Altai population was 31 % for the 3rd day and 52 % for the 6th day of cultivation. After a month of growing

R. rosea on 0.6 % agar, 3–4 shoots developed, and the shoot base thickened (Fig. 1c).

According to the literature data, *R. rosea* seeds exhibit variable morphological features, heterogeneous quality, low germination rate (7–24 %) and energy (up to 2 %) (Kim, 1999).

The choice of the explant tissue for *in vitro* introduction is crucial for successful realization of the morphogenetic potential of plant cells. Various types of explants were used for *R. rosea* propagation: leaves and leaf disks, axillary buds, shoot segments, and renewal buds of the rhizome (Dimitrov et al., 2003, Yin et al., 2004; Tasheva, Kosturkova, 2010). Apical buds of *in vitro* seedlings of *R. rosea* were also studied (Kirichenko et al., 1994). The possibility of *in vitro* propagation was shown for other representatives of the genus *Rhodiola* – *R. crenulata, R. yunnanensis, R. fastigata* and *R. sachalinesis* – using different types of explants (Hai-jun et al., 2006). Shoot segments were the most efficient explant type for *R. crenulata*, the leaf explant showed the best results for the other three species.

We have shown no additional pre-sowing treatment is required for effective *in vitro* germination of *R. rosea* seeds. The seedlings of *R. rosea* exhibit a high germination rate and are a promising explant type for *in vitro* mass propagation of the plant.

***In vitro* propagation**

At the stage of micropropagation, the medium was supplemented with cytokinins, alone or in combination with auxins, to remove apical dominance and to produce the largest number of shoots. We studied the effect of cytokinins BAP, zeatina, TDZ and auxin NAA on shoot production of *R. rosea* (Table).

Table

Effect of plant growth regulators on *in vitro* shoot production of *Rhodiola rosea*

Plant growth regulators, µM		Number of shoots/explants
Control		1
BAP	1	1.9 ± 0.6
	5	4.6 ± 1.2
	10	6.2 ± 1.5
TDZ	1	2.3 ± 0.8
	5	6.2 ± 1.3
	10	3.5 ± 0.9
Zeatin	1	2.2 ± 1.0
	5	4.1 ± 0.8
	10	6.5 ± 1.4
BAP (5) + NAA (2.5)		6.3 ± 1.1

The studies showed that a high concentration of cytokinins BAP or zeatin (10 µM) promoted *R. rosea* shoot formation, however, a negative process of shoot hyperhydratation was observed. Addition of 5 µM TDZ to culture medium triggered *R. rosea* budding but caused the formation of shortened shoots. Addition of 5 µM BAP and 2.5 µM NAA to culture medium resulted in an increased propagation coefficient of *R. rosea* (6.3 ± 1.1) compared to the control and in formation of shoots of standard morphology (Fig. 2).

The plant genotype and the combination and concentration of growth regulators in the medium are important for *in vitro* regeneration in various representatives of the genus *Rhodiola*. Thus, differences in ecotypes appeared to be an important factor influencing the processes of efficient callogenesis and organogenesis of *R. rosea* and *R. iremelica* (Ishmuratova, 1998). The optimal concentration of the cytokinin BAP for the Altai *R. rosea* were 10–15-fold higher for induction of *in vitro* cultures from immature leaves explants from a Tibetan golden root (Yin et al., 2004). The combination of 2.5 mg/l BAP and 0.1 mg/l NAA was found to be optimal for *R. crenulata* and *R. yunnanensis* and enhanced shoot formation by 71 and 84 %. The medium containing higher concentrations of auxin NAA (0.5 mg/l) at a similar BAP concentration was more appropriate for propagation of *R. fastigata* and *R. sachalinesis*, and the regeneration was 80 % (Hai-jun et al., 2006).

The mineral composition of the medium and the combination and concentration of growth regulators significantly affect callus formation, organogenesis, and regeneration. Various compositions of culture media – Murashige and Skoog (MS), Linsmaer and

Skoog, Gamborg, White, Nitsch and Nitsch – have been previously tested for *R. rosea*. The medium is typically supplemented with various combinations and concentrations of growth regulators. The most commonly used regulators for cultivation of *R. rosea* are BAP, α-indoleacetic acid (IAA), NAA, IBA, and 2.4-dichlorophenoxyacetic acid (2.4-D). The effect of zeatin, 2-izopentiladenin (2-iP), kinetin and TDZ was studied as well (Tasheva, Kosturkova, 2012).

***In vitro* rooting**

The success of *in vitro* technologies depends to a large extent on the stage of microshoot rooting. Regeneration of roots in different varieties and species differs and depends on both the plant ability to perceive rooting factors and the rooting methods used. Efficient rooting on the medium containing IBA was observed for *R. fastigata* and *R. sachalinesis* (87 % and 73 %, respectively). The auxin at a concentration of 2 mg/l was found to be efficient for *R. rosea* (Tasheva, Kosturkova, 2010).

At the stage of rhizogenesis, we observed 95–100 % rooting for microshoots of the selected forms of *R. rosea* on both hormone-free ½ MS medium and media supplemented with IBA (Fig. 2b). The average number of roots was 6.5 ± 1.9 per plant.

***Ex vitro* acclimatization**

Various substrates are used for acclimatization of the representatives of the genus *Rhodiola*. For example, a substrate containing soil, peat, and sand at a ratio of 3 : 1 : 1 ensured acclimatization of 70 %

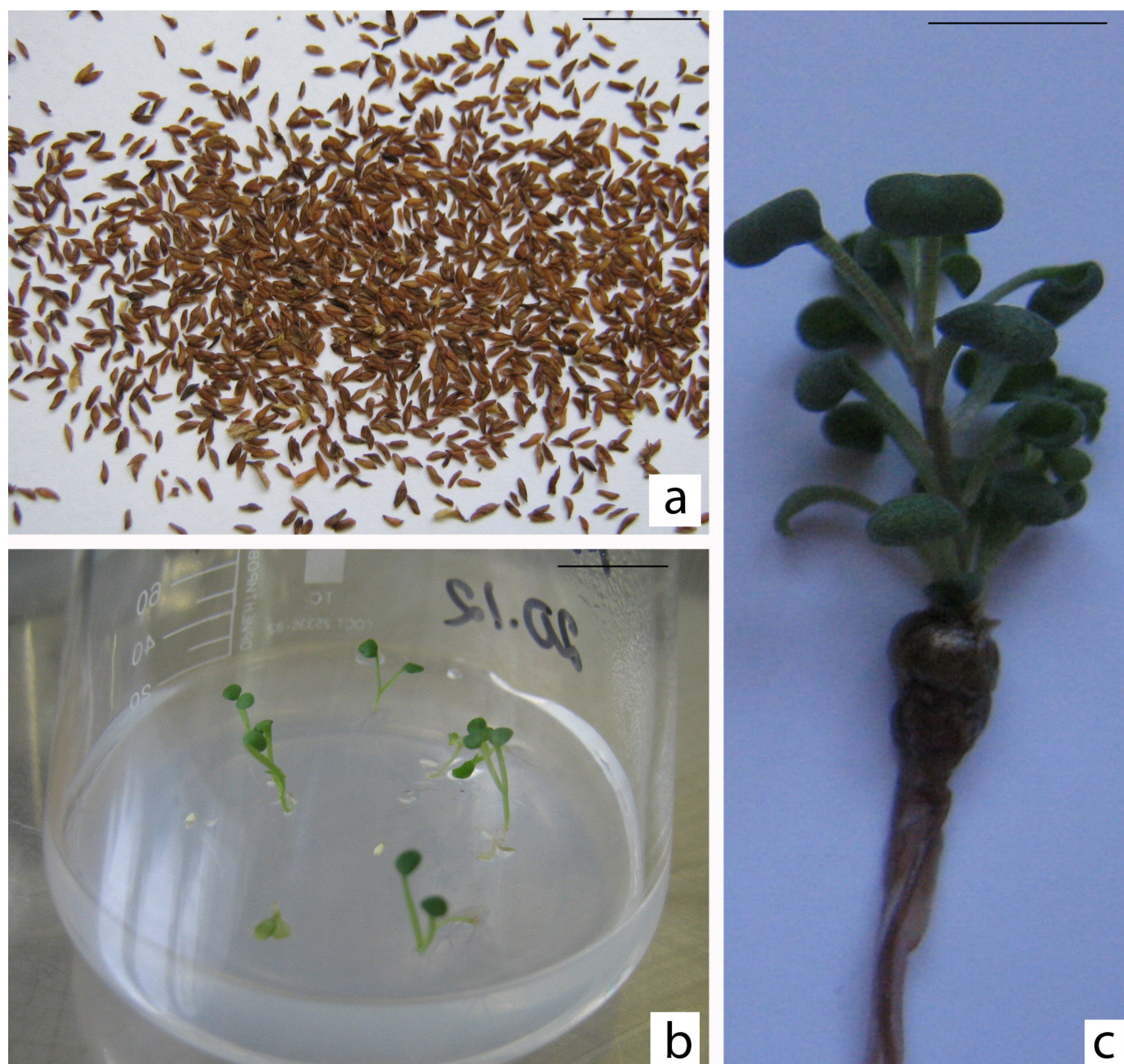

Fig. 1. *Rhodiola rosea*: a – seeds; b – seedlings on 0.6 % agar after 10 days of cultivation; c – seedlings on 0.6 % agar after 30 days of cultivation. Bar: 1 cm.

Fig. 2. *In vitro* cultivation of *Rhodiola rosea*: a – on MS medium supplemented with 5 μM BAP and 2.5 μM NAA; b – on ½ MS medium.

Fig. 3. Acclimatized plants of *Rhodiola rosea*: a – after 1 month of cultivation in cassettes; b – root system washed from the substrate and part of the rhizome with renewal buds.

of *R. rosea* plants and 66 % of *R. fastigata* plants (Hai-jun et al., 2006).

Acclimatization of *R. rosea* plants was carried out in the substrate consisting of perlite and peat at a ratio of 1:2 that ensured 78–84 % acclimatization (Fig. 3). In this substrate, the root system was observed to develop well, and the subsequent transplantation was carried out without disturbing the roots.

The average weight of roots (wet weight) during acclimatization and growth (7 months) was 12.2 ± 2.4 g. According to the literature data, the mass of *R. rosea* roots under natural conditions was 0.12–0.42–1.66 g in one-two-three-year plants, respectively. The root mass in cultivated plants (propagation by seeds) was 1.19–11.01–52.41 g, respectively. It was noted that *R. rosea* plants retain characteristics typical of natural populations when transferred and grown in culture medium. The ontogenetic development rate in cultivated plants was higher than that in plants growing under natural conditions. Cultivated plants exhibited an increased size of the aboveground and underground parts, accelerated growth of underground parts and their changed structure. Cultivated plants significantly exceeded wild-growing individuals in biomass; the share of caudex and roots was about 80 % of the plant weight (Platikanov, Evstatieva, 2008; *Rhodiola rosea*…, 2015).

We have shown that *in vitro* cultivation of *R. rosea* plants accelerates plant development. Seven-month-old plants of *R. rosea* cultivated in accordance with the biotechnology technique elaborated in the present study correspond to two-year-old plants grown by traditional reproduction techniques from seeds with respect to the parameters of the development of the aboveground part and biomass of the rhizome with roots.

Efficiency of the developed technique was as follows: seed germination – 52 %, sterility of explants – 100 %, propagation coefficient at the stage of shoot multiplication – 6.3 pieces per explant, the number of rooted microshoots – 95 %, the number of roots per plant – 6.5 ± 1.9 pieces per explant, and the number of acclimatized plants – 78 %.

The amount of the planting material that can be obtained based on the quantitative characteristics of the developed protocol for *R. rosea* plant propagation was calculated by the formula:

$$N \times M^{y} \times 0.95,$$

where N is the number of initial explants, M is the propagation coefficient, y is the number of subcultivations, and 0.95 is the rooting coefficient.

The calculations showed that one plant can produce 400000 *in vitro* grown plants a year. Taking into account the percentage of plant acclimatization to *ex vitro* conditions (78 %) and the time required for completing of the growth cycle, 312000 seedlings can be obtained from one plant within 2 years.

Conclusion

R. rosea or golden root is a valuable medicinal plant, the populations of which are significantly reduced due to uncontrolled harvesting. Biotechnology techniques, in particular, clonal micropropagation, enable *in vitro* propagation of the plant material and make the basis for the development of industrial plantations for *ex situ* conservation of *R. rosea*. The developed technique of clonal micropropagation and its optimization make it possible to obtain the planting material of high quality. Taking into account propagation coefficients, more than 300000 plants of *R. rosea* can be cultivated within a year. The plants cultivated *in vitro* exceed those grown by traditional methods in growth parameters, which helps to reduce the cycle of seedlings production from two to one year.

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In our study *in vitro* material from collection No. USU_440534 “Collection of living plants indoors and outdoors” was used.

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