In vitro micropropagation of wild rare plant Rhododendron ledebourii Pojark

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Abstract. Rhododendron ledebourii Pojark. is a highly decorative rare species of the flora of Siberia (Russia) and can be used as a source of biologically active substances, as well as in landscaping. We have optimized the in vitro propagation protocol for this species. Sterilization of annual shoots of field plants with 5% lysoformin-3000 provided 70% aseptic viable explants. The addition of 2 mg L\(^{-1}\) glycine to Anderson's medium in combination with 8 mg L\(^{-1}\) 2-isopentyladenine and 3 mg L\(^{-1}\) indolyl-3-acetic acid stimulated a high multiplication rate and active growth of axillary and adventitious shoots. The adaptation of regenerants in a hydroponic installation containing ¼ of the basic composition of macro- and microsalts according to Murashige and Skoog's medium promoted the development of powerful roots for subsequent successful acclimatization to ex vitro conditions. This approach ensured the survival rate of 90% microplants.

1. Introduction
The gene pool of living organisms is the treasure of the planetary level. The loss of any species or its population is an irreparable damage to the biological diversity of the Earth. Therefore, its preservation is a priority task for all states, as well as the most important area of modern science. The conservation of rare and endemic plant species of the wild flora is of particular interest. At the same time, many plant species that are under threat of destruction are considered as the main source of crop improvement, potential raw materials for the production of pharmaceuticals, functional foods and nutraceuticals, as well as cosmetic and perfumery products [1-2].

There are two main ways to solve the problem of preserving the diversity of the flora. The first way is in situ conservation, which is aimed at creating specially protected natural areas and preserving ecosystems in general. The second one is ex situ conservation of endangered species in the collections of botanical gardens. The efficiency of ex situ conservation of the plant gene pool can be increased by creating plant genetic banks [3]. In a number of countries, collections of cells, organs and plants cultivated in vitro have been formed and are effectively functioning [4-6]. At the junction of biotechnology and wildlife protection, the modern science “Plant Conservation Biotechnology” appeared, which provides additional methods for managing the genetic resources of rare plants [7]. The development of methods for clonal micropropagation is the basis for the creation of in vitro genetic banks of rare and endangered plant species, as well as one of the promising areas for the conservation of biodiversity in general [8]. However, despite the publication of a number of materials devoted to the conservation of rare and endangered plant species using micropropagation technologies, many of these species have not been introduced into in vitro culture, remaining poorly studied or not studied at all.
The genus *Rhododendron* L. (Ericaceae) comprises more than 1200 species and 10,000 varieties. In Russia, 16 species have been described, 13 of which grow in its Asian part, covering the areas of Siberia and the Far East. These species are frost-resistant, able to grow on weakly acidic soils, and successfully adapt when introduced in the south of Western Siberia. Most of them are highly decorative and can be used as components of landscape design. In addition, Asian species are promising sources of biologically active compounds with various medicinal properties, including antioxidant activity [9]. One of the most promising natural species is *Rhododendron ledebourii* Pojak., in the leaves and stems of which various phenols, essential oils, and vitamins are found [10-12]. Under natural conditions, it grows in the south of Western Siberia (Altai), in Eastern Siberia (the Sayan Mountains, Transbaikalia), in the Russian Far East and Mongolia [13]. *Rh. ledebourii* has the status of a Rare Species and is included in the Red Book of the Altai Territory (Russia). There are six known locations of this species in the region. The population size ranges from 2000 to 5000 specimens [14]. *Rh. ledebourii* is a winter-hardy evergreen shrub, reaching a height of 2 m. It grows on rocky mountain slopes, along the banks of mountain rivers, singly or in thickets in coniferous and larch forests. The flowering period is from May to June. In some years, secondary flowering is possible at the end of August. Due to the polymorphism of the species, the color of the corolla can vary from pink-lilac and pink to white.

The classical methods of propagation by seeds and cuttings are limited by the lack of stock plants, low seedling survival and poor rooting rate of cuttings. These difficulties are forcing scientists to develop new approaches for mass production of healthy planting stock. Clonal micropropagation can help to meet these challenges when utilizing wild flora in conservation and landscape projects. Studies carried out in different countries have revealed contradictory results both at the stage of initiation of in vitro culture and at the stage of micropropagation of rhododendrons. This fact significantly complicates or makes it impossible to repeat the described positive experience [15-20]. Probably, the main reason for such failures is the genotypic dependence of morphogenetic processes in tissue culture in all plant species, including rhododendrons. The aim of this study was to optimize the protocol for in vitro micropropagation of a rare species of the Altai flora, *Rh. ledebourii*.

2. Materials and methods

Young annual shoots, 15–20 cm length, were cut from adult plants introduced in the Altai Regional Ecological Center (Barnaul, Russia) from the wild population (the Altai Mountains, Russia). They were collected in mid-May during the period of active growth of mother shrubs, as well as at the end of August, when the secondary growth of young shoots was observed. The explants (cuttings 1–1.5 cm length with one axillary bud) were pre-washed by sequential immersion in soapy water (10 min) and running tap water (30 min). They were then rinsed in 70% ethanol for 30 seconds and sterilized in 3 or 5% (w/v) lysoformin-3000 for 20 minutes. After washing in sterile distilled water (three times for 5–10 min), aseptic explants were placed vertically in 100 mL glass jars with a semi-solid culture medium (20 mL).

In vitro initiation of primary shoots was carried out on Woody Plant Medium (WPM) [21] containing 2% (w/v) sucrose, 0.4% (w/v) agar, 2 mg L⁻¹ 2-iP (2-isopentyladenine), and 0.5 mg L⁻¹ IAA (indolyl-3-acetic acid). After 6 weeks, axillary shoots were transferred on micropropagation medium. We investigated three variants of culture media. They are Anderson's Medium (AM) [22], Murashige and Skoog's Medium (MS) [23] and WPM containing 3% sucrose and 0.5% agar. The basic media were supplemented with glycine (Gly) at concentrations of 1, 2, and 3 mg L⁻¹, IAA (3 mg L⁻¹) and 2-iP (8 or 10 mg L⁻¹). Duration of cultivation is 4–5 weeks for one reproduction cycle. For rooting, shoots with a length of at least 1.5–2 cm were transferred on ¼ WPM supplemented with 2% sucrose, 0.4% agar, 1 mg L⁻¹ IBA (indole-3-butyric acid), 500 mg L⁻¹ charcoal. The cultures were grown at 25±2°C, relative humidity 55–60%, 16 h photoperiod (day) with light intensity 35–55 µmol m⁻² s⁻¹ provided by cold white fluorescent lamps.

The acclimatization of regenerants included two stages. First, microplants cleaned from the nutrient medium were placed in a hydroponic installation containing ¼ of the basic composition of macro- and
microsalts according to MS. Then the plants were planted in pots with sphagnum moss and placed on racks in a phytotron at 26±2°C, relative humidity of at least 80%, and 16 h photoperiod.

The experiment was performed in three repetitions, at least 30 explants per variant. Statistical data processing was carried out using Microsoft Office Excel 2007 software. To compare the means, the LSD test was used (p = 0.05).

3. Results

The sterilization schemes used in our experiment turned out to be quite successful. Specimens collected in the spring were less infected (figure 1a, b). The highest yield of aseptic primary explants was 82% when using 5% lysoformin-3000. However, some of them were not viable. In vitro cultures were induced from 69% aseptic axillary buds (figure 2). Reducing the concentration of the sterilizing agent to 3% provided a better survival rate for cuttings (78%). However, taking into account the lower yield of decontaminated explants, it should be recognized that 5% solution was more effective.

Figure 1. Stages of in vitro propagation of Rhododendron ledebourii Pojark.: (a) an adult plant with young shoots (May); (b) annual shoots collected for the preparation of primary explants; (c) development of axillary buds after 2 weeks of in vitro culture; (d) development of axillary buds after 3 weeks of in vitro culture; (e) micropropagation on AM + 2 mg L⁻¹ Gly + 8 mg L⁻¹ 2-iP; (f) micropropagation on AM + 3 mg L⁻¹ Gly + 8 mg L⁻¹ 2-iP; (g) micropropagation on AM + 2 mg L⁻¹ Gly + 10 mg L⁻¹ 2-iP; (h) micropropagation in a hormone-free WPM.

The initiation of bud development was noted after 4–5 days of cultivation on nutrient medium (figure 1c). Within 2–2.5 weeks, the shoots reached a height of 1–1.5 cm (figure 1d). Their development took place within 5–6 weeks. Further proliferation of new shoots occurred mainly due to the induction of adventitious buds on seedling hypocotyls.

Optimization of the stage of micropropagation revealed the advantages of the AM, regardless of the concentration of Gly and 2-iP (table 1). The number of shoots per explant varied from 1.6 to 10.5 pieces, while the maximum number of regenerants on WPM reached only 5.6 pieces / explant. The MS medium turned out to be somewhat more productive, providing regeneration of up to 7.4 shoots per explant. The addition of Gly to the medium at a concentration of 2 mg L⁻¹ ensured the most complete realization of the morphogenic potencies of the explants during their cultivation both on AM (figure 1e) and MS medium. However, increasing the dose of the amino acid to 3 mg L⁻¹ led to a sharp
decrease in the frequency of regeneration of new shoots (figure 1f), especially in combination with 10 mg L\(^{-1}\) 2-iP. The reproduction rate was reduced to 1.6–1.9.

![Image](image_url)

**Figure 2.** The effect of sterilization on the survival of explants depending on the time of sample collection (May, August) (LSD\(_{0.05}\) for aseptic explants 6.2; LSD\(_{0.05}\) for surviving explants 8.1).

Comparative analysis of the effect of 2-iP on the micropropagation of *Rh. ledebourii* showed that its higher concentration in the medium (10 mg L\(^{-1}\)) led to the formation of vitrified regenerants, despite the high reproduction rate in some variants (AM + 2 mg L\(^{-1}\) Gly; MS + 2 mg L\(^{-1}\) Gly). Such plants quickly lost chlorophyll, acquired a violet hue or turned yellow, and during subsequent transplantation practically did not initiate de novo shoots (figure 1g). Cultivation of cuttings on hormone-free media, regardless of the base composition, blocked the development of axillary buds. In rare cases, a single shoot proliferated (figure 1h).

**Table 1.** The effect of culture medium components on the success of clonal micropropagation of *Rhododendron ledebourii* Pojark. (number of shoots / explant, pcs.).

| Gly, mg L\(^{-1}\) | 2-iP, mg L\(^{-1}\) | Base culture medium (hormone-free) |
|-------------------|------------------|-----------------------------------|
|                   | AM | WPM | MS |
| 0                 | 1.2 | 1.6 | 1.2 |
| 1                 | 5.1 | 1.8 | 4.5 |
| 2                 | 4.9 | 2.9 | 3.9 |
| 3                 | 10.5 | 5.6 | 7.4 |
|                   | 8.4 | 5.3 | 7.2 |
|                   | 3.2 | 1.6 | 4.9 |
|                   | 1.6 | 1.9 | 1.7 |
| LSD\(_{0.05}\)    | 1.3 | 0.9 | 1.2 |

Shoots were rooted on WPM with a reduced concentration of all components. Decreasing the agar dose to 0.4% increased the level of aeration, which promoted root proliferation. The stage lasted 7–8 weeks. About 80% of the shoots have developed a good root system (2–4 roots / regenerant). Adaptation of microclones in cassettes in a hydroponic installation lasted 2.5–3 weeks. During this period, the plants have adapted fairly well to ex vitro conditions. To maintain high humidity, the plants were covered with a transparent film for the first 5–7 days. The nutrient solution was changed every 8–10 days. Under such conditions, the plants increased the shoot length by an average of 60%, and the number and length of roots more than doubled.
This stage of acclimatization significantly increased the survival rate of plants when transferred to the substrate (figure 3a). After 2–3 months, about 90% microclones, transplanted into pots with peat, are formed as planting material of high quality (figure 3b, c).

**Figure 3.** Acclimatization of *Rhododendron ledebourii* Pojark. regenerants: (a), (b) rooting of regenerants in pots with sphagnum peat; (c) plants with a well-developed root system before planting in the greenhouse.

### 4. Discussion

In vitro propagation technologies contribute to the conservation of rare and endangered species of woody plants, as well as to the mass production of planting material for landscaping. For some species and varieties of rhododendrons, effective protocols have been developed, characterized by a wide variability in the composition of culture media and in cultivation conditions [15-20]. Seeds [24], flowers [25], leaves, tips of shoots [20], axillary buds, nodal shoot segments [26] are used to initiate in vitro culture. Micropropagation of rhododendrons through the development of axillary buds and the induction of adventive shoots is the most promising method for successful reproduction of these plants [17]. We succeeded in initiating a high frequency in vitro culture of a rare species of the Altai flora *Rh. ledebourii* using segments of annual shoots of field plants. For the first time, the chlorine-containing lysoformin-3000 was used as a sterilizing agent for rhododendron explants. This approach ensured the proliferation of 70% of viable buds.

The development of axillary shoots of evergreen rhododendrons is most often stimulated by cytokinins 2-iP [22], zeatin [16], and thidiazuron [18-20; 24]. The addition of auxins promotes the growth of the initiated shoots. For example, the ratio of 2-iP to IAA is usually 4 : 1. We used 2-iP in combination with IAA for micropropagation of *Rh. ledebourii*, but changed their ratio to 2.5: 1. Adding the amino acid glycine (2 mg L⁻¹) to the culture medium increases stress resistance of microplants. This fact, in turn, increased the multiplication coefficient and the viability of microshoots. The basic composition of nutrient media for in vitro cultivation of rhododendrons is also very diverse. AM, MS, WPM were used most often [15; 18; 20; 22; 24]. Schenk-Hildebrand's [26] and Economou-Read's [17] culture media were used less frequently. We tested three variants of culture media and found that AM was the most optimal for micropropagation of *Rh. ledebourii*. WPM is well suited for rooting and MS provides high adaptability in a hydroponic setup.

The adaptation of microplants to ex vitro conditions is the most critical stage in clonal micropropagation for most plant species. Regenerants, as a rule, are characterized by low photosynthetic capacity, weak activity of the stomatal apparatus, poorly developed chlorenchyma, and a low amount of cuticular wax. In addition, there is a high species-specificity of the survival rate of adapted plants in open ground conditions. One of the solutions to the problems is the use of hydroponic installations. Replacing the substrate with a liquid medium and controlling the supply of nutrient solution (automatic mode) provides high productivity when creating large volumes of planting
material. This approach provided a high survival rate of microplants (90%) of Rh. ledebourii and their further successful acclimatization.

5. Conclusion
Our study allowed us to optimize the protocol of in vitro reproduction, adaptation and acclimatization of a rare species of the Altai flora, Rh. ledebourii. Sterilization of annual shoots of field plants with 5% lysoformin-3000 provided a high yield of aseptic viable explants. We recommend collecting material in May during the period of active growth of young shoots. The use of AM supplemented with 2% sucrose, 3 mg L\textsuperscript{-1} IAA in combination with 8 mg L\textsuperscript{-1} 2-iP and 2 mg L\textsuperscript{-1} glycine stimulated a relatively high in vitro multiplication of axillary and adventive shoots and their active growth. Microplants were successfully acclimatized in a hydroponic installation and were formed well-developed root systems. These conditions of adaptation provided high survival rate of regenerants when planted in the ground. The described protocol can be used to create an in vitro collection and production of planting material of a highly decorative rare species Rh. ledebourii.

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