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In vitro propagation of *Lonicera tolmatchevii* (Caprifoliaceae), regressive relict endemic of the Sakhalin Island

Yu. E. Sabutski[1, 3]*, K. S. Berdasova[1, 4], A. S. Pianova (Berdasova)[1, 5], V. V. Sheiko[2, 6], A. V. Salokhin[1, 7]

[1] *Botanical Garden-Institute FEB RAS, Makovskogo St., 142, Vladivostok, 690024, Russian Federation*

[2] *Sakhalin Branch of Botanical Garden-Institute FEB RAS, Gorkogo St., 21, Yuzhno-Sakhalinsk, 693023, Russian Federation*

[3] *E-mail: alixar2006@yandex.ru; ORCID iD: https://orcid.org/0000-0003-3815-6142*

[4] *E-mail: k.berdasova@mail.ru; ORCID iD: http://orcid.org/0000-0003-1597-0846*

[5] *E-mail: berdasova_as@mail.ru; ORCID iD: http://orcid.org/0000-0002-7344-7589*

[6] *E-mail: viktorsheiko@mail.ru; ORCID iD: http://orcid.org/0000-0001-8042-656X*

[7] *E-mail: al-xv@mail.ru; ORCID iD: https://orcid.org/0000-0002-4902-8472*

**Corresponding author*

Keywords: Clonal micropropagation, cultivation conditions, rare species, Tolmachev's honeysuckle.

Summary. The protocol for clonal micropropagation of the Sakhalin Island endemic *Lonicera tolmatchevii* Pojark. has been developed for the first time, including stages of introduction into culture, reproduction, rooting under *in vitro* conditions and acclimatization under *ex vitro* conditions. It was shown that explants obtained from three-month-old seedlings actively form axillary shoots on Murashige-Skoog medium with the addition of cytokinins (BAP, Kn) and no formation of adventitious shoots occurred. It was found that the most effective combination of growth regulators for micropropagation of *Lonicera tolmatchevii* is a combination of 2 mg/L BAP and 0.5 mg/L IBA which leads to the formation of an average of 9.9 new shoots with active growth. The use of BAP without auxins leads to a decrease of the multiplication factor. The use of NAA at a concentration of 1 mg/l together with cytokinins (BAP, Kn) only resulted in the formation of callus that did not have morphogenic activity. The resulting regenerated plants were rooted successfully on hormone-free Murashige-Skoog medium with half the macronutrients. It has been shown that using auxins (IBA, IAA) at a concentration of 1 mg/L complicates the rooting process through the formation of callus tissue at the base of the explant. Rooted plants can be easily acclimatized to *ex vitro* conditions using a mixture of peat and vermiculite in a 1 : 1 ratio.

Размножение регрессивного реликтового эндемика острова Сахалин *Lonicera tolmatchevii* (Caprifoliaceae) в условиях *in vitro*

Ю. Е. Сабуцкий[1], К. С. Бердасова[1], А. С. Пьянова (Бердасова)[1], В. В. Шейко[2], А. В. Салохин[1]

[1] *Ботанический сад-институт ДВО РАН, ул. Маковского, д. 142, г. Владивосток, 690024, Россия*

[2] *Сахалинский филиал Ботанического сада-института ДВО РАН, ул. Горького, д. 21, г. Южно-Сахалинск, 693023, Россия*

Ключевые слова: жимолость Толмачёва, клональное микроразмножение, редкий вид, условия культивирования, *Lonicera tolmatchevii*.

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Аннотация. Впервые разработан протокол клонального микроразмножения эндемика острова Сахалин *Lonicera tolmatchevii* Pojark., включающий введение в культуру, размножение, укоренение в условиях *in vitro* и акклиматизацию в условиях *ex vitro*. Показано, что экспланты, полученные от трёхмесячных сеянцев, активно образуют пазушные побеги на среде Мурасиге-Скуга с добавлением цитокининов (БАП, Кн), при этом образование адвентивных побегов не наблюдалось. В ходе экспериментов было найдено, что наиболее эффективной комбинацией регуляторов роста для микроразмножения *Lonicera tolmatchevii* является сочетание 2 мг/л БАП и 0,5 мг/л ИМК, приводящее к образованию в среднем 9,9 новых побегов с активным ростом. Применение БАП без ауксинов приводит к снижению коэффициента размножения. Использование НУК в концентрации 1 мг/л совместно с цитокининами (БАП, Кн) приводило только к образованию каллуса, не обладающего морфогенной активностью. Полученные растения-регенеранты успешно укореняются на безгормональной среде Мурасиге-Скуга с половинным содержанием макроэлементов. Показано, что применение ауксинов (ИУК, ИМК) в концентрации 1 мг/л затрудняет процесс корнеобразования посредством образования каллусной ткани в основании экспланта. Укорененные растения легко проходят акклиматизацию к условиям *ex vitro* при использовании смеси торфа и вермикулита в соотношении 1 : 1.

Introduction

Lonicera tolmatchevii Pojark. (Tolmachev's honeysuckle) is a regressive relict endemic of the Russian Far East flora (Nedoluzhko, 1983; Sheiko, Taran, 2017). It grows only in the central and northwestern parts of the Sakhalin Island, in the middle reaches of the Tym River and in the lower reaches of its tributaries. The plant is found singly or in small groups in floodplain bushes and on sandy-alluvial deposits. *Lonicera tolmatchevii* is a deciduous shrub, up to 1.8 m high, with rather large elliptical leaves (up to 12 cm in length and 7.5 cm in width), glandular tetrahedral shoots, lemon-yellow flowers and black spherical fruits. Despite the high germination rate of seeds (about 90 %), the main method of reproduction in nature is vegetative, which is directly depends on the growing conditions and the strong increase in the Tym River level after the rains (Sheiko, 2018). The limiting factors for this species are the fragmentation of small micropopulations, poor seed reproduction, low competitive ability in relation to shrubs and grasses (the main competitor in the habitat is *Swida alba*), as well as the threat of destruction during the roads and pipelines construction.

The total number in nature is low and, according to estimates, does not exceed 500 specimens (Sheiko, 2019). As a vulnerable species, *Lonicera tolmatchevii* is included in the Red Book of the Russian Federation (Firsov, 2024), the Red Book of the Sakhalin Region (Sheiko, 2019), but unfortunally, it is absent in specially protected natural areas.

Tolmachev's honeysuckle also can be interesting as an ornamental plant. The large bright green leaves and small height of the shrub give the greatest value and make it suitable for planting in groups (Sheiko, 2018). Flowers and fruits have a moderate decorative effect. It is known that the plant is winter-hardy, adapted to the short and cool northern summer, but is still extremely rare (Firsov et al., 2017). Currently, *Lonicera tolmatchevii* is grown in botanical gardens and arboretums in eight Russian cities: Moscow, St. Petersburg, Pereslavl-Zalessky, Cheboksary, Yoshkar-Ola, Abakan, Rodniki (Ivanovo Region), and Yuzhno-Sakhalinsk. In Moscow, St. Petersburg and Yuzhno-Sakhalinsk it is completely winter-hardy (Firsov et al., 2017; Sheiko, 2018; Trusov et al., 2023).

Today, there is a large number of works devoted to the *in vitro* micropropagation of various honeysuckle species, but there is no information on the propagation of *Lonicera tolmatchevii*, what can be explained by the difficulty of obtaining material for such studies. Also, there is no data on *in vitro* propagation of the closest species, North American twinberry honeysuckle (*L. involucrata*). In the literature the process of micropropagation has been studied more detailed for *Lonicera caerulea*, its subspecies and varieties, widely used as a berry crop (Karhu, 1997a; Dziedzic, 2008; Sedlak, Paprstein, 2008; Zapolsky et al., 2018; Kadhim et al., 2019; Orlova et al., 2021). The purpose of this work is to study the cultivation factors' influence on the process of micropropagation of the rare endemic species *Lonicera tolmatchevii*, as well as to develop methods for rooting *in vitro* and adaptation to *ex vitro* conditions, which would allow preserving, reproducing and more widely introducing this rare species into culture.

Materials and methods

Objects. For obtaining a sterile culture *in vitro* we used mature seeds of *Lonicera tolmatchevii* as the starting material. Seeds were collected in the

Sakhalin Branch of Botanical Garden-Institute FEB RAS (Yuzhno-Sakhalinsk). Three-month-old seedlings were used as a source of explants to study the micropropagation stage.

Reagents and equipment. $AgNO_3$ (LenReaktiv, Russia) was used as plant material sterilizing agent. All work on material sterilization and subcultivation was carried out under aseptic conditions in a laminar flow hood BAVnp-01-"Laminar-S"-1.5 (LORICA, Russia). Explants were cultivated in a nutrient medium according to the Murashige and Skoog (MS) (Murashige, Skoog, 1962) with the addition of 6-benzylaminopurine (BAP), kinetin (Kn), 2-iso-pentenyladenine (2-iP), indolylbutyric acid (IBA), indolylacetic acid (IAA), α-naphthylacetic acid (NAA), vitamins (Sigma-Aldrich, USA), 3 % sucrose (Helikon, Russia) and 0.6 % agar-agar (Sigma-Aldrich, USA). Culture media (pH = 5.7) were autoclaved using a steam sterilizer MLS-3781L (Sanyo, Japan) at 121 °C for 20 min. Vitamins and growth regulators were added after autoclaving. For *in vitro* cultivation foil-sealed tubes (20 × 200 mm) containing 10 ml of medium were used. Cultivation was carried out at a temperature of 24–26 °C, relative air humidity of 70 % and illumination of 2000–3000 lux with a photoperiod of 16 hours.

Statistical processing. During morphometric parameters analysis of the developing microshoots, the following parameters were determined: number (pcs.), length of shoots (cm) and multiplication factor (number of shoots per explant). In each case of the experiment at least 20 explants were used, the experiment was repeated 2–3 times. Statistical data processing was carried out according to standard methods using the Microsoft Office software (Excel 2010).

Method Execution

Introduction to *in vitro* culture

1. *Sterilization of seed material*. Before sterilization, the seeds were pre-soaked in water for 24 hours and then washed in 0.1 % Tween 20 solution for 20 minutes. Sterilization was carried out in a laminar flow hood using 1 % $AgNO_3$ (20 min), followed by washing with sterile distilled water (3 times for 2 min). The sterilization efficiency was 97 %.

2. *Cultivation of seedlings*. The seeds were germinated and cultivated on hormone-free MS medium throughout the experiment. Germination occurred in the light (large-scale after 14 days) and reached 55 %. Three-month-old seedlings containing an average of 4 internodes were used for the experiment.

Clonal micropropagation stage

1. *Explants*. At the micropropagation stage, stem segments of the resulting seedlings were divided in half (containing 2 nodes) and used as explants. During explants cultivating the main process was the axillary shoots development or callus formation at the point of contact with the medium. The formation of adventitious shoots was not observed. Subsequent subculturing of the explants onto a new medium of the same composition was carried out every 4 weeks.

2. *Composition of the nutrient medium*. At the micropropagation stage, MS nutrient medium with a full content of micro- and macrosalts was used. To study the micropropagation process 11 variants of media containing different concentrations of cytokinins (1 and 2 mg/L 2-iP, 1 and 2 mg/L BAP, 2 mg/L Kn), as well as combinations with auxins (2 mg /L BAP + 1 mg/L NAA, 4 mg/L BAP + 1 mg/L NAA, 2 mg/L BAP + 0.5 mg/L IAA, 2 mg/L BAP + 0.5 mg/L IBA, 2 mg/L Kn + 1 mg/L NAA, 10 mg/L Kn + 0.1 mg/L NAA) were used. The obtained data about plant growth regulators effect on development of explants is shown in Table 1.

According to available literature for the micropropagation of honeysuckles, Murashige and Skoog medium is the most commonly used with the addition of BAP at a concentration of 0.5–4 mg/L or its combination with auxins such as IBA and IAA (Boonour et al., 1988; Karhu, 1997a; Dziedzic, 2008; Sedlak, Paprstein, 2008; Osburn et al., 2009; Zapolsky et al., 2018; Kadhim et al., 2019; Orlova et al., 2021). Other available cytokinins and auxins also were applied in our research. During the experiment, it was noted that the explants' cultivation in media containing BAP or Kn with 1 mg/L NAA leads only to the callus emergence without additional shoot formation (Table 1). The literature describes methods for micropropagation of genus *Lonicera* representatives through induction and subsequent cultivation of the resulting callus tissue (Cambecedes et al., 1991; Ochatt, 1991; Wang et al., 2009; Trang et al., 2016). However, the calli obtained in our experiments did not give any morphogenic response when further cultivated on the same media or transferred to a medium with BAP or IBA at a concentration of 1 mg/L.

Analysis of the obtained data after explants' cultivation for 90 days in propagation media (Table 2)

showed that using only cytokinins (Kn, 2-iP and BAP) gives a small multiplication factor and, often, weak growth of the resulting microshoots. The use of Kn and 2-iP at concentrations of 1 and 2 mg/L leads to the formation of an average of 2.1–2.3 shoots per explant, for which weak growth and partial necrosis are observed during cultivation for more than 3 months. The best shoot growth with cytokinins is observed only in the case of a BAP applying (Fig. 1A, 1B). Application of BAP at a concentration of 1 mg/L leads to an increase the multiplication factor to 5.4 shoots per explant (Fig. 1A), and to 6.2 at a concentration of 2 mg/L.

Table 1. Influence of growth regulators on *Lonicera tolmatchevii* explants' development (60 days of cultivation)

Exp. number	Contents of growth regulators, mg/L						Morphogenic response
	2-iP	BAP	Kn	IBA	IAA	NAA	
1	1	–	–	–	–	–	Shoots
2	2	–	–	–	–	–	Shoots
3	–	–	2	–	–	–	Shoots
4	–	1	–	–	–	–	Shoots
5	–	2	–	–	–	–	Shoots
6	–	2	–	–	–	1	Callus
7	–	4	–	–	–	1	Callus
8	–	2	–	0.5	–	–	Shoots
9	–	2	–	–	0.5	–	Shoots and callus
10	–	–	2	–	–	1	Callus
11	–	–	10	–	–	0.1	Shoots
12	–	–	–	–	–	–	Shoots

Table 2. Influence of growth regulators on *Lonicera tolmatchevii* explants' micropropagation (90 days of cultivation)

Exp. number	Growth regulators	Average shoot number, pcs	Average shoot length, mm
1	1 mg/L 2-iP	2.12 ± 1.00	9.31 ± 0.72^{c}
2	2 mg/L 2-iP	2.31 ± 0.92	11.28 ± 0.70^{c}
3	2 mg/L Kn	2.11 ± 0.88	9.13 ± 0.64^{b}
4	1 mg/L BAP	5.40 ± 0.72^{b}	16.02 ± 0.78^{c}
5	2 mg/L BAP	6.18 ± 0.70^{b}	18.43 ± 1.18^{d}
8	2 mg/L BAP, 0.5 mg/L IBA	9.89 ± 0.32	20.07 ± 0.60^{b}
9	2 mg/L BAP, 0.5 mg/L IAA	3.22 ± 0.80^{c}	7.54 ± 0.52^{a}
11	10 mg/L Kn, 0.1 mg/L NAA	4.62 ± 0.82^{c}	15.27 ± 1.28^{d}
12	Hormone-free	1.20 ± 0.42^{a}	5.11 ± 1.30

Various letters indicate significant difference values for each column ($p \leq 0.05$).

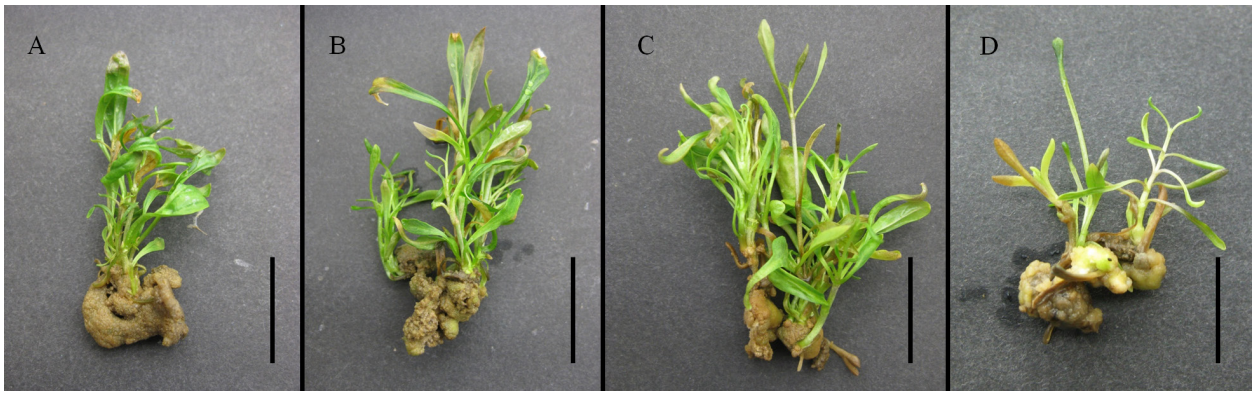

Fig. 1. The influence of the most effective growth regulators' combinations on micropropagation of *Lonicera tolmatchevii* explants: 1 mg/L BAP (A); 2 mg/L BAP (B); 2 mg/L BAP + 0.5 mg/L IBA (C); 10 mg/L Kn + 0.1 mg/L NAA (D). Ruler interval: 1 cm.

When cytokinins were used together with auxins, active formation of new shoots occurred only in the case of combinations of 2 mg/L BAP and 0.5 mg/L IBA (Fig. 1C), as well as 10 mg/L Kn and 0.1 mg/L NAA (Fig. 1D). The multiplication factors for these experiments were 9.9 and 4.6, respectively. The shoots obtained in these cases were also characterized by active growth, what allows to use them for the rooting stage *in vitro*. Application of 2 mg/L BAP and 0.5 mg/L IAA combination led to weak shoot growth (multiplication factor – 3.2) and strong callus formation. Probably, IAA can also be used to stimulate the formation of microshoots but at a lower concentration.

3. *Rooting and adaptation to non-sterile conditions*. After 3 months of cultivation on propagation media the resulting regenerated plants were divided into individual shoots and transferred to MS medium with half the macronutrients without growth regulators and media containing 1 mg/L IBA or IAA. According to the literature, microshoots of genus *Lonicera* representatives take roots relatively easily on MS medium with reduced to 1/2 or 1/3 content of macrosalts with the addition of auxins (IBA or IAA) at a concentration of 0.8–5 mg/L (Karhu, 1997b; Dziedzic, 2008; Sedlak, Paprstein, 2008; Wang et al., 2009; Zapolski et al., 2018; Kadhim et al., 2019). During an experiment on the rooting of *Lonicera tolmatchevii* microshoots, it was found that cultivation on a hormone-free medium leads to the appearance of roots already by the 10th day. After 40 days, the explants had a well-formed root system (Fig. 2A), and rooting efficiency reached 100 % in some replicates. On medium containing auxins, only occasionally cases of root formation were observed even after three months of cultivation. This process was accompanied by the formation of callus at the base of the explant, which probably had a negative effect on root formation.

To adapt rooted microshoots to *ex vitro* growing conditions, a mixture of peat and vermiculite (1 : 1) was used as a substrate. After 90 days of cultivation on the medium (Fig. 2B) plants were washed from the medium and planted in a sterile substrate for rooting. Under these conditions the plants developed well and quickly grew new shoots (Fig. 2C).

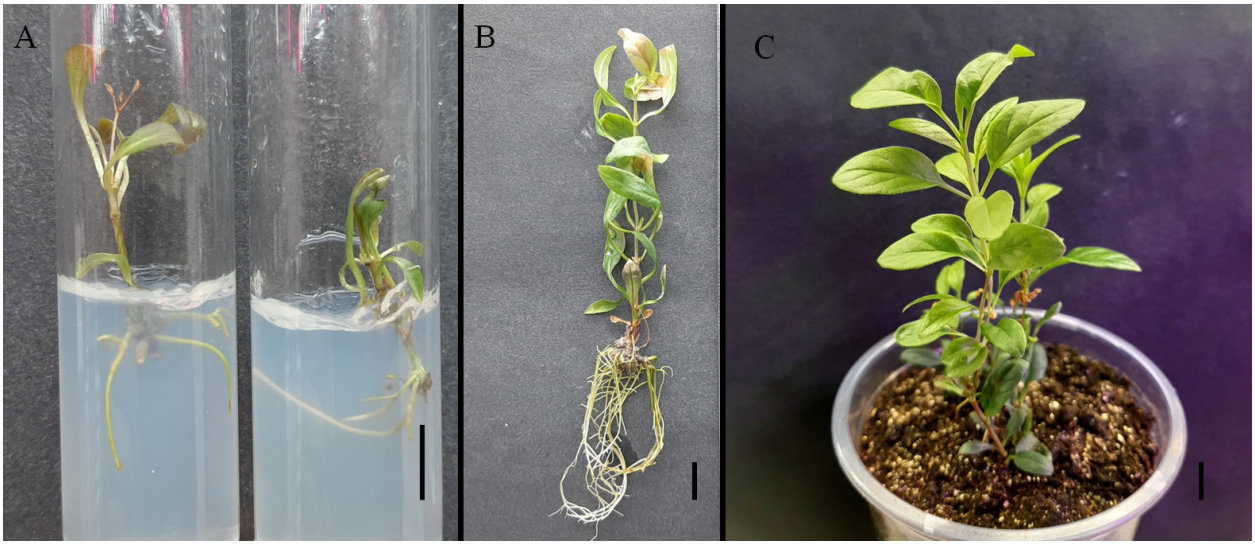

Fig. 2. Roots development of *Lonicera tolmatchevii* microshoots on ½ MS medium without growth regulators for 40 days (A) and 90 days (B); acclimatized *Lonicera tolmatchevii* plants to *ex vitro* conditions (C). Ruler interval: 1 cm.

Conclusion

During the study, an effective approach for clonal micropropagation of the Red Book endemic of the Sakhalin Island *Lonicera tolmatchevii* was developed for the first time. It has been shown that explants obtained from seedlings *in vitro* have a high regenerative potential and when cultivated on MS medium with the addition of cytokinins actively form axillary shoots. In the course of the experiments, it was found that the most effective combination of growth regulators for micropropagation of *Lonicera tolmatchevii* is a combination of 2 mg/L BAP and 0.5 mg/L IBA leading to the formation of an average of 9.9 new shoots with active growth, what allows their further rooting and acclimatization *ex vitro*. The resulting regenerated plants successfully rooting on hormone-free MS medium with half

the macronutrients while the addition of 1 mg/L IBA or IAA leads to the formation of callus at the base of the explants and inhibited the process of root formation. Rooted plants can easily acclimatize to *ex vitro* conditions using a mixture of peat and vermiculite in a 1 : 1 ratio. The developed protocol for micropropagation of *Lonicera tolmatchevii* can be used for the conservation, wider distribution and introduction into culture of this rare species as an ornamental plant.

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