



In vitro propagation of *Eranthis stellata* (Ranunculaceae), endemic species with narrow distribution in the Russian Far East, Northeast China and North Korea

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ABSTRACT

Eranthis stellata (Ranunculaceae) was cultured *in vitro* for the first time, for which the use of peduncles with underdeveloped flower buds as primary explants was found to be effective. Upon introduction to culture *in vitro*, explants should be cultured in the dark at low temperature (17°C) to prevent phenolic oxidation. A culture medium containing 1/2 MS supplemented with 5 µM 6-benzylaminopurine is optimal for the propagation of this species. A morphogenic response rate of 32 % was achieved, and the propagation rate amounted to 4.2 shoots per explant.

Key words: *in vitro* culture, *Eranthis stellata*, micropropagation, conservation

РЕЗЮМЕ

Эрст А.А., Эрст А.С. Размножение в культуре *in vitro* *Eranthis stellata* (Ranunculaceae), эндемичного вида с ограниченным распространением на Дальнем Востоке России, Северо-Востоке Китая и в Северной Корее. Впервые введен в культуру *in vitro* *Eranthis stellata*, сем. Ranunculaceae. Показана эффективность использования в качестве первичных эксплантов цветоносов с недоразвитыми бутонами. Отмечено, что на этапе введения в культуру *in vitro* экспланты необходимо культивировать при пониженной температуре (+17°C) в темноте для преодоления фенольного окисления. Питательная среда 1/2 MS, дополненная 5 µM 6-бензиламинопурина, является оптимальной для размножения данного вида: морфогенный ответ составил 32 %, коэффициент размножения 4.2 шт./эксплант.

Ключевые слова: культура *in vitro*, *Eranthis stellata*, микроразмножение, сохранение

Three species of the genus *Eranthis* Salisb. are recorded in Russia: *E. sibirica* DC., *E. stellata* Maxim., and *E. tanboensis* Erst (Malyshev 2005, Erst et al. 2020). The range of *E. stellata* includes Northeastern China, North Korea, and Far Eastern Russia, where it grows in waterlogged forest valleys, coniferous and coniferous-deciduous forests, and floodplain meadows near forest rivers and streams. The species is common in Primorye Territory and occurs sparsely in adjacent areas. It is listed in the Red Books of the Amur Region (Darman 2009) (as *Shibateranthis stellata* (Maxim.) Nakai), Jewish Autonomous Region (Yakubov 2006), and Khabarovsk Territory (Babkina & Safonova 2008) and is referred to as category 3G – a rare species found locally in the western range.

Similar to many other geophytes, species of the genus *Eranthis* have great potential as ornamental plants. For example, the European species *E. hyemalis* (L.) Salisb. shows a high rate of vegetative propagation by tubers and is widely cultured (Marcinkowski 2002). Mayorov & Vinogradova (2013) classify *E. hyemalis* as a potentially invasive species. However, according to available literature data, the renewal rate of white-flowering *Eranthis* is not high under conditions of introduction. For example, *E. sibirica* is considered unsuitable for introduction to the territory of Siberia (Sobolevskaya 1984, Semenova 2007).

Seed propagation of the representatives of the genus *Eranthis* is restricted due to morpho-physiological dormancy of seeds associated with underdeveloped embryos, which

are characteristic of many representatives of the family Ranunculaceae (Baskin & Baskin 1986, Bullowa & Ozeri 1975, Forbis & Diggle 2001). For example, a 4-month cold stratification is required to germinate the seeds of *E. hyemalis* and *E. longistipitata* Regel (Tipirdamaz & Gomurgen 2000, Erst & Erst 2019). The seeds of *E. stellata* are found to rapidly lose the germinative ability of freshly harvested seeds, and after 2 months of storage in the laboratory, they completely lose germinative ability (Nesterova 2004).

All species of *Eranthis* are rare and endemic, and their renewal rate is quite low. Therefore, effective methods of propagation and conservation of these species should be developed, including biotechnological methods. No methods of *Eranthis* propagation *in vitro* has been developed previously. However, many studies report the potential of this method for the propagation and conservation of geophytes (Ziv & Lilien-Kipnis 2000, Ozcan et al. 2007, Cig & Basdogan 2015, Podwyszynska 2015, Muraseva & Novikova 2018).

The choice of explants to initiate aseptic culture is the first stage and one of the most important stages of propagation *in vitro*. The use of underground plant organs (rhizome buds, bulbs, tubers) often causes high culture contamination (Seyring 2002, Cig & Basdogan 2015), but they are still some of the most common types of explants used for the introduction of geophytes to culture *in vitro*. It is the underground organs of geophytes that have renewal buds. They are rich in storage compounds, which contribute to their high regenerative ability.

Notably, the underground organs of geophytes are also easy to store and transport, which is also important in work with rare and poorly studied plant species. Nevertheless, a number of studies have shown that the use of above-ground organs, such as floral elements, leaves, and shoots, is more preferable for introducing rare and endangered plant species into culture. Using these parts helps to avoid complete removal of the mother plant from natural habitats (Ziv & Lilien-Kipnis 2000, Muraseva & Novikova 2018) and to prevent the high contamination that is characteristic of underground organs (Seyring 2002).

The aim of this study is to investigate the morphogenetic potential of various types of explants of *E. stellata* for introduction and propagation *in vitro*. The data are reported for the first time. The study results could improve development of systems of species propagation and conservation.

MATERIAL AND METHODS

E. stellata plants were collected from their natural range in the Far East in Vladivostok, Russia, in the Malaya Sedanka River Basin on 16.05.2019.

Tubers. Plant material was introduced to culture after the growing season of *E. stellata* (May 2019). The tuber surface was sterilized by first rinsing it with running water to remove soil, followed by washing in 5 % Tetranil U solution (Delia-Sept) for 5 min. The tubers were immersed in 20 % sodium hypochlorite solution (Domestos) in a laminar box for 5 min, removed, and scraped with a medical bandage. The material was then placed in 70 % alcohol for 30 s and after immersed in 0.2 % HgCl₂ solution for 20 min. The surface sterilization was completed by rinsing three times with sterile distilled water.

Sterile tubers were separated into parts or placed as a whole on agarized media with the following composition: Murashige and Skoog (MS) medium (Murashige & Skoog 1962) supplemented/with 1 μM 6-benzylaminopurine (BAP) and 0.1 μM α-naphthaleneacetic acid (NAA) (Appli-Chem, Germany); MS supplemented with 1 μM thidiazuron (TDZ); Gamborg and Eveleigh (B5) medium (1968) supplemented with 1 μM BAP and 0.1 μM NAA; and B5 supplemented with 1 μM TDZ. The culture media were supplemented with 10 mg/L of AgNO₃ to prevent phenol oxidation. The cultures were incubated at a constant temperature of 24±2°C under 16 h of daily exposure to illumination of 3000 lux from cool-white fluorescent lamps.

Unexpanded flower buds and peduncles. *Eranthis* tubers were germinated in moist sphagnum moss in a refrigerator at 7°C. Flower buds emerged after 3 months. The

surfaces of peduncles bearing flower buds were sterilized in 0.2 % HgCl₂ solution for 20 min, followed by rinsing three times with sterile distilled water. Next, the explants were separated into parts: the upper part of the peduncle with a flower bud, the flower bud, and the upper part of the peduncle without a flower bud. The culture media contained ½ MS + BAP 0.25 μM and 1/2 MS + TDZ 0.25 μM and were supplemented with 10 mg/L AgNO₃ to prevent phenolic oxidation.

At the micropropagation stage, we used ½ MS culture medium supplemented with 5 μM BAP or 5 μM TDZ. At the stage of introduction to culture, explants were placed in a thermostat in the dark at 17±0.5°C for 3 weeks. The explants were then cultured under standard conditions (temperature 24±2°C, photoperiod 16/8)

RESULTS

We improved the method of surface sterilization for the underground organs of *E. stellata*, and the average yield of sterile explants was 78 %. However, *in vitro* culture of the underground organs caused tissue necrosis and death of explants on all the studied culture media, including culture media supplemented with AgNO₃.

For floral organs, the yield of sterile material was 100 %. Upon introduction of this type of explant to *in vitro* culture, we showed that only peduncles bearing flower buds induced a morphogenic response under the chosen culture conditions. The growth of the peduncle base and initiation of adventitious buds could be observed on culture media supplemented with 0.25 μM BAP or TDZ, and 0.25 μM TDZ+AgNO₃ 10 mg/L (Table 1, Fig. 1). The most distinct morphogenic response was noted on the medium supplemented with BAP – 36 %.

At the stage of introduction to *in vitro* culture, we used the method of explant culture in the dark at low temperature (17°C) to minimize the negative effects of phenolic oxidation. The method was chosen based on our preliminary experiments, in which standard conditions (24±2°C, 16/8 photoperiod) caused the death of explants before the onset of a morphogenic response. Peduncles and flower buds without peduncles did not develop on the studied culture media, and browning and death of explants were observed after 4 weeks of culture.

After 8 weeks of culture, the explants were transferred to media for mass propagation. The introduction of BAP to the culture medium evoked a greater morphogenic response than when TDZ was introduced at a similar concentration (Table 2). At the same time, the propagation factors did not differ significantly in the studied culture media.

Table 1. Morphogenic response of various types of explants of *Eranthis stellata* at the stage of introduction to culture *in vitro*, % (n=10)

Medium variants	Flower bud	Peduncle	
		without a flower bud	with a flower bud
½ MS +0.25 μM BAP	0	0	36
½ MS +0.25 μM BAP +10 mg/l AgNO ₃	0	0	0
½ MS +0.25 μM TDZ	0	0	12
½ MS +0.25 μM TDZ + 10 mg/l AgNO ₃	0	0	10

Table 2. Growth and development parameters of *Eranthis stellata* explants at the multiplication stage. LSD test: means denoted by the same letter were not significantly different (P≤0.05).

Medium variants	Morphogenic response, %	Mean number of shoots per explant
½ MS + 5 μM BAP	32 ^a	4.2±0.9 ^a
½ MS + 5 μM TDZ	8 ^b	3.6±1.2 ^a

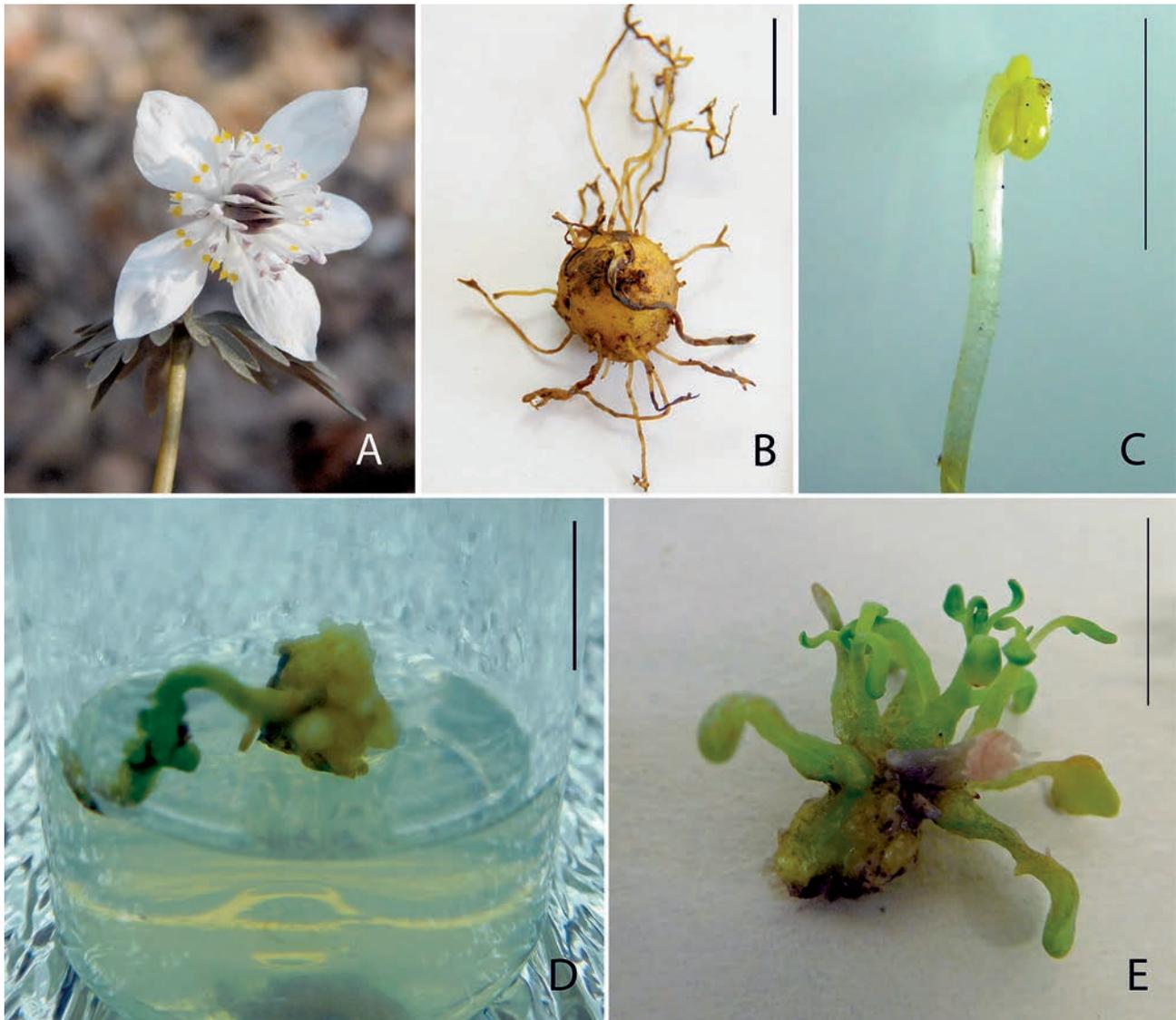


Figure 1 *Eranthis stellata* Maxim. *in vivo* and *in vitro*: A – flowering plants (photo by V.V. Yakubov); B – tuber at the end of the growing season; C – peduncle with a closed wrapper bud; D – adventive shoot formation at the base of the peduncle on a nutrient medium $\frac{1}{2}$ MS supplemented with $0.25 \mu\text{M}$ BAP; E – the development of microshoots on a $\frac{1}{2}$ MS nutrient medium supplemented with $5 \mu\text{M}$ BAP. Bar: 1 cm

DISCUSSION

Various types of explants are used to introduce geophytes to culture *in vitro*. At the initial stages of culture, studies are mainly hindered by high contamination (Wang & van Staden 2001), tissue browning due to phenolic oxidation (Buchheim & Meyer 1992), and callus development, which prevents the initiation and development of meristematic zones (Li et al. 1984). In our study, when using *E. stellata* tubers as primary explants, we faced the problem of significant phenolic tissue browning. The potential of using parts of *E. stellata* tubers can be evaluated only after selecting antioxidants that inhibit or minimize the negative effects of phenolic browning on explant tissue in culture *in vitro*. It should be noted that representatives of the family Ranunculaceae have been successfully propagated *in vitro* from buds of the underground organs, such as *Thalictrum daltzellii* Hook., *Paeonia lactiflora* Pall., and *Helleborus* species (Dhooghe & Van Labeke 2007, Sharanappa & Ravishankar Rai 2011, Shen et al. 2012).

The use of peduncles and unexpanded flower buds as primary explants proved to be an effective technique for the introduction of *E. stellata* into culture. Peduncles have been successfully used to propagate geophytes such as *Hippeastrum* spp. hybrids, *Allium*, *Dichetostemma*, *Eucrosia*, *Gladiolus*, and *Haemanthus* (Seabrook & Cumming 1977, Ziv & Lilien-Kipnis 2000). Plant material typically exhibits a high regenerative capability at the early stages of development. Peduncles harvested before differentiation of the reproductive organs or at the stage of active growth are a promising source of primary explants (Ziv & Lilien-Kipnis 2000). In our study, we showed the effectiveness of using peduncles with an underdeveloped flower bud.

Some plants are characterized by axillary buds on the peduncle, and they can be successfully propagated using meristems, as in the case of *Levisia cotyledon* (S. Wats.) B.L. Robins (George & Tripepi 2004). In most cases, peduncles do not have meristems, so morphogenesis proceeds similarly to the formation of the *de novo* structure. Apical and axillary buds

can be effectively used since a stimulus (such as a growth regulator) is sufficient for their development, whereas explants without a meristem require preliminary formation of meristematic zones or somatic embryogenesis.

At the initial stages of culture, we placed floral explants in the dark at low temperature to minimize the negative effects of phenolic browning of *E. stellata* tissues. For the *in vitro* culture of *Phalaenopsis*, it was shown that the total phenol content and browning rate increased when the temperature was increased from 20 to 30°C (Zhao et al. 2006). It was found that explant culture in the dark at 5°C reduces the phenolic tissue browning and increases explant viability at the introduction stage of shoots of *Malus pumila* Mill. cv. Fuji and *Pyrus bretschneideri* Rend., cv. Jinhua to culture *in vitro* (Wang et al. 1994).

We have shown that the morphogenic response of *E. stellata* explants was greater on culture media supplemented with BAP than that on media supplemented with TDZ. BAP alone or in combination with auxins is the most commonly used cytokinin for geophyte propagation (Cig & Basdogan 2015). For example, BAP was successfully used for *in vitro* propagation of *Gladiolus* species and varieties (Hussey 1977; Akhare et al. 2008), and BAP in combination with auxins was used to propagate *Muscari mirum* Speta (Nasircilar et al. 2011), *Iris stenophylla* Hausskn & Siehe ex Baker subsp. *allisonii* B. Mathew (Nasircilar et al. 2011), and species of the genus *Fritillaria* (Kulkhanova et al. 2015, Muraseva et al. 2015, Muraseva & Novikova 2018).

CONCLUSION

Thus, the morphogenic potential of various types of explants of *E. stellata* was investigated for introduction and propagation *in vitro*. The use of peduncles bearing underdeveloped flower buds as primary explants was found to be effective. It was shown that explants should be cultured in the dark at low temperature (17°C) to minimize phenolic browning of tissues at the stage of introduction to culture *in vitro*. The most distinct morphogenic response was observed for ½ MS culture medium supplemented with 5 µM BAP. The results of this study could enhance the development of the clonal micropropagation protocol for *E. stellata* to develop comprehensive measures using advanced techniques aimed at *ex situ* preservation of *E. stellata* populations.

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