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In Vitro Propagation and Conservation of Rare Species *Fritillaria meleagris* L. from Floral Explants

D. S. Muraseva, T. I. Novikova, and A. A. Erst

Central Siberian Botanical Garden, Siberian Branch, Russian Academy of Sciences, ul. Zolotodolinskaya 101, Novosibirsk, 630090 Russia

e-mail: dsmuraseva@csbg.nsc.ru

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Abstract—The peculiarities of the in vitro regeneration of the rare species *Fritillaria meleagris* L. from floral explants have been studied for the first time. Upon the establishment of in vitro culture and during the multiplication stage, the most effective is the B5 nutrient medium supplemented with 0.44 μM BAP, 3.22 μM NAA, and 2.28 μM IAA. During the multiplication stage, the regeneration rate reaches 80% and the number of bulblets per explant is 3.9 ± 0.3. It is established that the morphogenesis of de novo formed structures depends on the mineral composition of the medium: the use of mineral-based B_5 leads to adventitious shoot formation (gemmogenesis), while the use of BDS causes morphogenic callus formation and the gemmorhizogenesis on its surface. The stimulating effect of low temperatures (+7°C) on bulblet development and adaptation to ex vitro conditions is noted.

Keywords: *Fritillaria meleagris* L., in vitro regeneration, floral explants, morpho-histological analysis, adventitious shoot formation, biodiversity conservation

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INTRODUCTION

The problems of plant diversity conservation arising from ecosystem destabilization caused by environmental pollution, anthropogenic load, an increase in the number of invasive species, and climate change require the search and development of new strategies. One effective approach to successfully preserving rare species under ex situ conditions is the use of biotechnologies (Engelmann, 2011). The processes of in vitro regeneration are the basis not only for the development of multiplication technologies, but also for basic research on plant morphogenetic development. One fundamentally important property of morphogenesis is the versatility of its ways under both in vivo and in vitro conditions; it makes it possible to explore the potential of plant cells and modulate the processes of development in the organs and tissues culture (Batygina, 2010).

Snake's head fritillary—*Fritillaria meleagris* L. (Fam. Lilliaceae) is a rare species with a disjunctive habitat. It is spread in Western Siberia and the European part of Russia, as well as in Eastern Europe, Kazakhstan, and the Mediterranean; it is listed in the Red Data Book of the Russian Federation (2008), with a status of 3c. Natural populations of this ephemeroid suffer from its bulbs being gathered and dug up due to the attractive color of its flowers and early blooming. The low rate of natural reproduction of *F. meleagris* is also one of the causes of the vulnerability of natural populations of this species. A number of studies were devoted to the conservation and propagation of snake's head fritillary, which proved the effectiveness of alternative biotechnological approaches instead of traditional breeding methods (Vechernina, 2004; Nikolic et al., 2008; Vetchinkina, 2010; Subotić et al., 2010; Petric et al., 2011, 2013; Jevremovic et al., 2010).

In vitro plant regeneration, particularly that of monocots, is often challenging (Laslo et al., 2011). Somatic cells of this class of higher plants are differentiated very early and then lose their mitotic activity and morphogenetic potential. Embryos or organs with meristematic zones are used most often for in vitro multiplication. However, the seeds of species of the genus *Fritillaria* have a deep morphophysiological type of dormancy that makes it difficult to use them as primary explants (Nikolaeva et al., 1999; Vetchinkina et al., 2012).

Other types of explants are used for in vitro culture initiation of fritillaries: bulbous scale, for example, for *F. verticillata* (Vechernina, 2004), *F. meleagris* (Laslo, 2011), and F. *thunbergii* (Paek, Murthy, 2002); leaf base for *F. meleagris* (Subotić et al., 2010); petals for *F. imperialis* (Mohammadi-Dehcheshmeh et al., 2007, 2008), and bulblets for *F. unibracteata* (Gao et al., 1999).

The use of floral organs as primary explants ensures the preservation of the mother plant, which is especially important when working with rare species; it makes it possible to avoid a high level of contamination that is typical for explants from the underground organs, and it is an alternative to the bulb scales that make up the bulb

of the donor plant, especially in the case of their limited number (1–3 scales) (Ziv and Lilien-Kipnis, 2000; Mohammadi-Dehcheshmeh et al., 2008).

It was found that the in vitro culture regeneration processes of the genus *Fritillaria* can occur in two ways: through somatic embryogenesis or gemmogenesis; the de novo formation of the structures may occur either directly or indirectly through a phase of callus formation (Paek and Murthy, 2002; Vechernina, 2004). According to the literature, somatic embryogenesis, which often has an intermediate stage of callus formation, is most typical for *F. meleagris* (Petric et al., 2010; Subotić et al., 2010). Since the ultimate aim of these works is to preserve the germplasm of rare and endangered species and to maintain it in a stable condition, an important point in the development of micropropagation techniques is to study the ways of in vitro morphogenesis.

The aim of this research is to study the morphogenesis of a rare species *F. meleagris* in the in vitro culture of petals as the basis for the development of highly efficient and stable systems of reproduction and conservation of the species.

MATERIALS AND METHODS

Closed flower buds of *F. meleagris* (totally, 37 buds) served as the starting material for its introduction to in vitro culture; they were taken in 2013–2015 from the plants growing at the introduction site of the Laboratory of Ornamental Plants of the Central Siberian Botanical Garden, Siberian Branch, Russian Academy of Sciences (Novosibirsk).

Preparation of an Aseptic Culture

Surface sterilization was performed by immersion into 20% aqueous solution of Domestos for 20 min, then the buds were washed three times with sterile distilled water. Sterile petals were separated from the receptacle and used as primary explants. The culture medium developed for *F. imperialis* was used at the stage of the introduction to in vitro culture (Mohammadi-Dehcheshmeh et al., 2008): mineral foundation by the prescription of Gamborg and Eveleigh (B_5) (Gamborg and Eveleigh, 1968) and growth regulators 0.44 μM 6-benzylaminopurine (BAP) in combination with 3.22 μM α-naphthaleneacetic acid (NAA) and 2.28 μM indole-3-acetic acid (IAA). The hormone free B_5 medium was used as a control.

In Vitro Propagation

Conglomerates of regenerated microbulblets were transferred to media for further propagation. Modified B_5 and Dunstan and Short (BDS) (Dunstan and Short, 1977) nutrient media supplemented with BAP, NAA, and indole-3-acetic acid (IAA) at concentrations of 0.2–10.0 μM were used at the stage of propagation. Plant material was cultivated under conditions of 16 h illumination/8 h darkness at 23 ± 2°C by standard methods (Kalinin et al., 1980). Subculture period was 35–40 days.

Morphohistological Analysis

A Stereo Discovery v. 12 stereo microscope (Carl Zeiss, Germany) was used for morphological studies. A detailed histological study of the processes of morphogenesis was carried out on permanent microscopic preparations prepared according to Pausheva (1988). The plant material was fixed in FAA–ethanol: formaldehyde: glacial acetic acid (100 : 7 : 7). Washing and further storage was performed in 70% ethanol. Thin sections were stained with hematoxylin according to Ehrlich with aniline blue tint. The histological analysis of plant development was carried out using an Axioskop-40 light microscope (Carl Zeiss, Germany) equipped with AxioCam MRc5 digital camera using AxioVision v. 4.8 software for receiving, processing, and image analysis.

Rooting and Adaptation to Ex Vitro Conditions

At the stage of rooting, the microbulblets were planted on B_5 medium with a twice-reduced content of macro- and microsalts supplemented with 5 μM NAA. All nutrient media for rooting contained particulate activated carbon (0.5 mg/L). To induce rhizogenesis, microplants were placed under low positive temperature in a light thermostat (Rumed, Germany) at 7°C for 1.5–2 months. Indicators of growth and development were recorded at the end of this period: the diameter of the bulb and the number of bulb scales and the number and the length of roots. 1/2 B_5 nutrient medium and 23 ± 2°C served as a control.

To adapt the regenerated plants to ex vitro conditions, they were planted in containers filled with a mixture of shredded coconut fiber and sand (3 : 1) closed with a film and transferred to the greenhouse.

Statistical Analysis of the Results

All experiments were carried out twice. The statistical analysis package Microsoft Excel was used to process the results. The diagrams show the average arithmetic values and confidence intervals. This paper discusses the differences significant at the 95% significance level.

RESULTS AND DUSCUSSION

When petals are used as primary explants, in addition to providing a high degree of sterility, it is necessary to reduce the damaging effect of the sterilizing agent. We developed the surface sterilization mode for flower buds using a 20-min exposure to a 20% solution of Domestos; it proved to be effective, and explant sterility reached 91–98%. The viability of primary explants was 76%.