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EX SITU CONSERVATION OF THE SPECIES *DIANTHUS MARTUNIENSIS, D. GROSSHEIMII* (*CARYOPHYLLACEAE*), ONOBRYCHIS ARAGATZI (*FABACEAE*) AND COTONEASTER HAJASTANICUS (*ROSACEAE*) BY THE CLONAL MICROPROPAGATION METHOD

Appropriate conditions were selected for clonal micropropagation of the rare species of Armenian flora, including: *Dianthus martuniensis, D.grossheimii (Caryophyllaceae), Onobrychis aragatzi (Fabaceae),* and *Cotoneaster hajastanicus (Rosaceae).*

Clonal micropropagation, rare species, Armenian flora, ex situ conservation

Нерсесян А. А., Щербакова Е. Н., Даниелян А. Г., Мелконян Н. Г., Навасардян Е. М. *Ex situ* сохранение видов *Dianthus martuniensis, D. grossheimii (Caryophyllaceae), Onobrychis aragatzi (Fabaceae)* и *Cotoneaster hajastanicus (Rosaceae)* методом клонального микроразмножения. Разработаны условия клонального микроразмножения редких видов флоры Армении *Dianthus martuniensis, D. grossheimii (Caryophyllaceae), Onobrychis aragatzi (Fabaceae)* и *Cotoneaster hajastanicus (Rosaceae)*.

Клональное микроразмножение, редкие виды, флора Армении, сохранение ex situ

Ներսեսյան Ա. Ա., Շչերբակովա Ե. Ն., Դանիելյան Ա. Հ., Մելքոնյան Ն. Գ., Նավասարդյան Ե.Մ.: *Dianthus martuniensis, D. grossheimii (Caryophyllaceae), Onobrychis aragatzi (Fabaceae)* և *Cotoneaster hajastanicus (Rosaceae)* տեսակների *ex situ* պահպանությունը կլոնալ միկրոբազմացման մեթոդի կիրառմամբ: Մշակվել են Հայաստանի հետևյալ հազվագյուտ տեսակների՝ *Dianthus martuniensis, D. grossheimii (Caryophyllaceae), Onobrychis aragatzi (Fabaceae), Cotoneaster hajastanicus (Rosaceae)* կլոնալ միկրոբազմացման պայմանները:

Կլոնալ միկրոբազմացում, հազվագյուտ տեսակներ, Հայաստանի ֆլորա, ex situ պահպանություն

Development of plant conservation tactic and strategy for the species that are under the threat of extinction is one of the urgent tasks of advanced botanical science. Application of clonal micropropagation techniques for plants serves the basis to establish *in vitro* genetic banks that would help to preserve genetic diversity *ex situ* (Belokurova et al., 2005; Demidchik et al., 2019; Doan et al., 2012; Zholobova et al., 2012; Molkanova et al., 2005; 2020a, 2020b).

The purpose of the performed study was to devise conditions for microclonal propagation of the rare species of Armenian flora – *Dianthus martuniensis* M.L. Kuzmina*, D.grossheimii S*chischk.*, Onobrychis aragatzi* Arevsch.*,* and *Cotoneaster hajastanicus* Nersesian.

Dianthus martuniensis is an extremely ornamental species of wild carnation with bright-pink flowers. In Armenia, this perennial plant is met within the Sevan and Darelegis floristic regions only and grows on rocks and stony slopes of the higher mountain belt. The total area of the species is delimited by the Southern Transcaucasus and the northeastern Anatolia.

The species *D. grossheimii* is included in the Red Data Book of Plants of Armenia (Tamanyan et al., 2010) under the EN category. This perennial wild carnation is an endemic plant of Armenia, growing in mountain steppe and meadow terrains of the higher mountain belt of central Armenia, in the Sevan and Darelegis floristic regions.

The endemic Armenian species *Onobrychis aragatzi* is a perennial sainfoin with whitish-pink flowers and densely pubescent fruits. It grows in the Aragats, Aparan and Yerevan floristic regions, in meadow-steppe, mountain steppe and open-woodland terrains spreading from the medium mountain belt to the alpine belt.

The species *Cotoneaster hajastanicus* is an Armenian endemic that grows on detrital soils, stony and rocky slopes of the higher mountain belt in the Upper-Akhourian, Shirak, Lori, Zanghezour and Meghri floristic regions of Armenia. This species of cotoneaster has prostrate branches and orange-red fruits with 4-6 pyrenes.

Material and Methods

Seeds of the studied species stored in the collection of the Seed Bank of Armenian Flora at the

Institute of Botany after A. Takhtajyan NAS RA, as well as collections of green parts of the plants (*Cotoneaster hajastanicus)* were used in the study. Juvenile plantlets produced from sterile-germinated seeds of the indicated species, and the apical meristem and axillary buds of the plants, served as explants introduced into an isolated culture. For the purposes of microclonal propagation, explant cultivation conditions best fit to initiate the morphogenesis and to provide the growth of test-tube plants were selected.

Shoots of *C. hajastanicus* with apical meristem and accessory buds were sterilized during 3 min in 70°alcohol, then for 25 min in a 0.1% diacid solution, and then washed in 4 courses with sterile water and planted on diverse nutrient media. Seeds of the studied species were first treated with 96°alcohol, washed with water and after sterilized – for 20 minutes in 15% hydrogen peroxide solution, followed with single course of washing in sterile water, and then for 20 min in 2.5% sodium hypochlorite solution, followed with triplicate washing in sterile water. At the next step, they were planted on a mineral medium to germinate. The produced sterile seedlings were transplanted onto various nutrient media based on the media of Muraschige and Skoog (MS) (Muraschige, Skoog, 1962) or White (1948; cited according to Kalinin et al., 1981:95). Depending on the specific tasks and test conditions set, vitamins (thiamine, pyridoxine, nicotinic acid) and hormonal substances (plant growth regulators, PGR) were added into the nutrient media in diverse concentrations and combinations: 6-Benzylaminopurine (BAP) at 0.2-2 mg/l, Kinetin at 0.2-1 mg/l, Indole-3-acetic Acid (IAA) at 0.5-2 mg/l, 1-Naphthaleneacetic Acid (NAA) at 0.5-1 mg/l, and Indole-3-Butyric Acid (IBA) at 0.5-1.5 mg/l.

Flasks with explants, as well as the produced mericlones, were kept in a climatic camera set at the photoperiod of 16/8 hours and the temperature of 26°/21°C. The growth of the mericlones had been sustained by periodic subculturing onto a fresh nutrient medium. Afterwards, plantlets that developed roots were displanted into the substrate of soil mixed with crushed tuff granules at the ratio of 5:2.

Studied Samples

Dianthus martuniensis: Armenia, Vayots Dzor Marz, above Elpin village, 1750 –1800 m a.s.l., N 39.82˚, E 45.13˚, 20.10.2018, A. Nersesyan, SBAF 1440.

Dianthus grossheimii*:* Armenia, Vayots Dzor Marz, Селимский перевал, в окрестностях Караван-сарая, 2150-2250 m a.s.l., A. Asatryan, 12.08.2015, SBAF 519/1.

Onobrychis aragatzi**:** Armenia, Aragatsotn Marz, Mt. Aragats, near Antarut village, 40°23’ N 44°16’, 2020 m a.s.l., 15.07. 2019, A. Nersesyan, SBAF 178/2

Cotoneaster hajastanicus**:** Armenia, Shirak Marz, Jajur Pass, stony slopes and screes, 2000 m a.s.l., N 40.88˚, E 43.99˚, 27.09.2019, A. Nersesyan, A. Danielyan, N. Melkonyan, SBAF 1581

Results of the Studies and Discussion

Dianthus martuniensis. After seed germination, embryo roots were cut from the sprouts off, and the upper parts were transferred onto the nutrient media containing mineral salts according to MS protocol and diverse sets and concentrations of hormonal compounds. It was observed that the nutrient medium containing 1 or 2 mg/l BAP and 0.5 mg/l of IAA promoted active growth of the apex of the sprout, which elongated up to 5-6 cm and formed 6 to 7 nodes. In the meantime, base of the cutting grew wide and produced adventive shoots, and axillary shoots started to grow concurrently (Fig.1). For the purposes of micropropagation, lateral shoots were trimmed, divided unto cuttings and moved onto fresh nutrient medium of the same composition. In such case, plant growth cycle lasts for 30-45 days. With the BAP concentration reduced to 0.5 mg/l, the growth of planted cuttings proceeded very slowly, developing not more than 2-3 nodes, and the cuttings turned yellow and died off shortly after that. In case Kinetin content of 0.5-1 mg/l was used in the nutrient medium instead of BAP, *D. martuniensis* plants elongated to 5-8 cm, but formed just 3-4 nodes, while axillary shoots did not form. If the auxin applied in the nutrient medium was represented by NAA at 0.5 mg/l, insignificant shoot growth was observed, but adventive shoots did not branch of the cutting base and growth of axillary shoots did not occur either.

Nutrient media with reduced contents of mineral salts and vitamins and no cytokinin are often used to initiate rooting in cuttings. In our tests with the

medium that contained 50% or 75% concentrations of mineral salts and vitamins, growth of shoots was insignificant whichever auxin had been added in the medium (IAA or NAA); however, in combination with NAA (0.5 mg/l), thin roots formed in abundance, but could be broken off easily in an attempt to take them out from agar. The best results were obtained with the use of 100% concentration of mineral salts and vitamins added with either NAA (0.5 mg/l), or IBA (1 mg/l). Just in 2 weeks, shoot apex and axillary shoots were observed to grow on such medium and roots formed at the base. To transfer the cuttings to the substrate, they had to be grown on filter paper supports in a liquid nutrient medium to avoid root damage. In that case, cuttings were transferred to the substrate together with the supports. First days after planting, the substrate had been watered with MS-based solution of mineral salts, and the plantlets were covered with caps to prevent drying. A few days after plantlets adapted to the new conditions the caps were removed (Fig.2).

Dianthus grossheimii. Sterile seedlings of *D. grossheimii* were transferred onto diverse MS-based nutrient media. Cuttings started to grow at 1 or 2 mg/l BAP content in the medium, combined with 0.5 mg/l of IAA, and callus that formed in their base was producing multiple adventive shoots. Concurrently, the 1st and 2nd order axillary shoots were forming. The shoots were thin and had small ring-like curled leaves. Total height of a plant was just 2-3 cm and it looked like a small "ball" or small "hedgehog" (Fig. 3). Reducing BAP concentration in the medium to 0.5 mg/l, it appeared possible to provide for plant growth optimum: 4 to 5 adventive shoots with well-developed axillary shoots were developing at the base. Total plant height ranged up to 5 cm and leaves were bigger. It was easy to separate such shoots one from another and to divide them into cuttings for micropropagation. In case 1 mg/l Kinetin concentration was applied as the cytokinin, cuttings of *D. grossheimii* grew up to 4-5 cm long, but did not develop adventive shoots, turned yellow and died off soon.

Nutrient medium with halved strength of mineral salts and vitamins and with auxin added only was used to induce rooting. It appeared that 1 mg/l concentrations of both IAA and IBA stimulated rooting, but as the roots were very thin, plantlets had to be cultivated first on filter paper supports placed in liquid nutrient medium before being transferred to the substrate. Two to three weeks after planting into the substrate and as the adaptation period ended, new shoots grew from the cutting base and the main plant died off (Fig. 4).

Onobrychis aragatzi. After seeds germinated, sterile juvenile plants were transferred onto diverse nutrient media. In the medium containing 2 mg/l BAP and 0.5 mg/l IAA, callus grew at the explant base and produced many short (1-1.5 cm) shoots with small leaves and very short petioles. In case BAP concentration was reduced to 1 mg/l, callus grew at the explant base as well, but adventive shoots were fewer, leaves were bigger and had long petioles (Fig.5). The formed shoots could be easily separated one from another for re-planting on a fresh nutrient medium. BAP concentration at 0.5 mg/l did not promote micropropagation: additional shoots did not develop on the planted cutting, leaf petioles grew longer and then the plant perished. Neither Kinetin nor NAA applied as cytokinins, or auxin were observed to promote shoot growth. Plants turned yellow and perished soon after they were planted on such media. It must be commented that in case of *O. aragatzi* plant growth period on the indicated media with 1-2 mg/l BAP comprises 22-25 days, after which plantlets shall be displanted on a fresh nutrient medium.

In case of *O. aragatzi*, MS-based nutrient media applied to initiate rooting had 50% concentration of mineral salts without vitamins or hormones, and with 0.5 -1 mg/l IAA or with 1-1.5 mg/l IBA. Small roots formed in the medium with IAA 15 days after planting and abundant rooting was observed in the medium with IBA after 30-35 days (Fig. 6). Plantlets were transferred to the substrate after adaptation period, when caps were removed from flasks, but plants were still kept in the agarized medium for 4-5 days.

Cotoneaster hajastanicus. Upon surface sterilization, young shoots having apical and 1 or 2 axillary meristems were planted onto MS nutrient media containing diverse sets and concentrations of vitamins and hormonal compounds. Multiple additional shoots formed at the cutting base and axillary shoots started to develop during month-long cultivation on the medium containing 2 mg/l BAP and 0.5 mg/l IAA. However, the shoots were short (1-1.5 cm) and had very small leaves. When BAP

concentration in the medium was reduced to 0.5-1 mg/l, additional shoots were still forming at the explant base, but axillary shoots developed in smaller numbers and had larger leaves (Fig.7). The substitute of BAP for Kinetin (1 mg/l) did not have impact in terms of explant growth.

Media applied to initiate rooting of shoots in case of C. *hajastanicus* included 1) MS medium with halved concentration of mineral salts and vitamins and 1 mg/l IBA, and 2) hormone-free White's medium with halved content of vitamins and sucrose. Rooting was observed in both types of media, however, on the White's medium it started as early as 10 days after cuttings were planted, and plantlets there were ready for replanting into the substrate on the 20th day. In the meantime, the process of rooting observed on the MS medium supplemented with IBA extended to 35-40 days. After being displanted to the substrate, plantlets adapted to external conditions very rapidly (Fig. 8).

Conclusions

1. MS medium containing 1-2 mg/l BAP combined with 0.5 mg/l IAA shall be applied for clonal micropropagation of *D. martuniensis*, and, to provide for the rooting of plants, it is possible to use either 0.5 mg/l NAA, or 1 mg/l IBA without cytokinin.
2. The best growth of shoots and micropropagation of *D. grossheimii* was observed on the MS medium containing 0.5 mg/l BAP and supplemented with 0.5 mg/l IAA. MS nutrient medium containing halved set of mineral salts and vitamins and either IAA, or IBA in the concentration of 1 mg/l shall be used to stimulate rooting.
3. To perform micropropagation and to get big number of shoots in case of *O. aragatzi*, it is recommended to use MS nutrient medium that contains BAP at the concentration of 1-2 mg/l combined with 0.5 mg/l IAA. To induce rooting, it is better to use IBA at1-1.5 mg/l.
4. MS nutrient medium containing 0.5-1 mg/l BAP combined with 0.5 mg/l IAA shall be used for micropropagation in case of *C. hajastanicus*. To induce rooting, it is better to use a hormone-free White's medium that contains halved content of vitamins and sucrose.

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Fig. 1. *Dianthus martuniensis* – mericlones

Fig. 2. *Dianthus martuniensis* – rooted plantlets

Fig. 3. *Dianthus grossheimii* – an isolated culture

Fig. 4. *Dianthus grossheimii* – plantlets

Fig. 5. *Onobrychis aragatzi* – adventive shoots

Fig. 6. *Onobrychis aragatzi* – rooting

Fig. 7. *Cotoneaster hajastanicus* – micropropagation

Fig. 8. *Cotoneaster hajastanicus* – displanted plants