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RESEARCH ARTICLE

In vitro Propagation of *Dianthus mainensis*, an Endemic Plant from the West Sayan (North Asia)

Anna A. Erst[1*], Andrey S. Erst[2] and Dmitry N. Shaulo[3]

1. Central Siberian Botanical Garden, Zolotodolinskaya st. 101, Novosibirsk 630090, Russia; Altai state university, Lenina st. 61a, Barnaul 656049, Russia.
2. Central Siberian Botanical Garden, Zolotodolinskaya st. 101, Novosibirsk 630090, Russia; Tomsk state university, Lenina st.36, Tomsk 634050, Russia. Email: erst_andrew@yahoo.com
3. Central Siberian Botanical Garden, Zolotodolinskaya st. 101, Novosibirsk 630090, Russia. Email: dshaulo@yandex.ru
** Corresponding author. Phone: +79236412467; Fax: +7(383)3301986; Email: annaerst @yandex.ru*

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ABSTRACT: The method of preservation and *in vitro* propagation of rare species *Dianthus mainensis* was offered. Seeds were used as starting material for *in vitro* propagation. Explants were cultured on MS medium supplemented with BAP and NAA. The greatest number of shoots was obtained when supplementing 3 μM BAP (5.5 shoots per an explant). This medium provided direct morphogenesis without appearance of somaclonal variants. Rooting of shoots *in vitro* observed when using half strength MS medium without growth regulators. Regenerants of *Dianthus mainensis* were successfully adapted and transferred to the experimental field.

KEY WORDS: Conservation, *Dianthus mainensis*, *in vitro* culture, micropropagation, rare taxa.

INTRODUCTION

The genus *Dianthus* L. belongs to the *Caryophyllaceae* Juss. family and includes 300–350 species distributed in Europe, Asia, Africa and North America (Gorshkova, 1936). The centre of genus diversity is in the Mediterranean area (Takhtajan, 1988). The section *Chinenses* Tzvelev is closely related *D. chinensis* L. Species, which are morphologically distinct and geographically isolated (De-Quan and Turland, 2001; Barkalov et al., 2006.). *Dianthus mainensis* Shaulo et A. Erst has been described in the Borus Ridge area in the West Sayan mountain system (Zjatkova, 1969). This mountain range is part of the Altai-Sayan floristic province (Takhtajan, 1988). The geographical location of the Western Sayan (in the centre of the Asian continent) and environmental conditions have directly influenced the taxonomic diversity of vascular plants. A distinctive feature of this region is an increased level of local endemism. The greatest number of rare taxa, including *Dianthus mainensis,* are limited to mountain elevations along the Yenisei river system, or the "Sayan Corridor" (Krasnoborov, 1976; Shaulo, 2006), with an area of approximately 100 km^2. The distribution of this stenotopic species is limited in that it grows on calcareous rocky substrates.

Dianthus mainensis is listed as an endangered species in the Red Book of the Republic of Khakassia (2012). Collecting seeds and live plants from any species in the Red Book of Russia is prohibited. Seed regeneration in species of the genus *Dianthus* is possible, but collecting the seeds of rare plants is difficult due to inaccessible habitats and singular growth. A possible method to protect endangered species is *in vitro* culture. Micropropagation methods have been developed and are currently used for endemic and ornamental *Dianthus* species (Mikulik, 1999; Paunescu and Holobiuc, 2003; Pareek et al., 2004; Ali et al., 2008; Holobiuc et al., 2010; Cristea et al., 2010). Conservation through modern biotechnology offers the possibility to rescue the natural heritage of plant biodiversity.

Several species of the genus *Dianthus* are very decorative and have gained attention for the purposes of breeding. With bright, attractive flowers, a high number of branching shoots, leaves with a bluish tinge and a long flowering period (May to August), *Dianthus mainensis* is a promising candidate for the selection process. Propagation *in vitro* is a rapid, cost-effective way to conserve species and facilitate mass propagation.

The aim of this study was to develop a clonal micropropagation method to conserve *Dianthus mainensis*. Our results may serve a guide for future research in this field, including plant conservation, repopulation programs and selection.

Fig. 1. *Dianthus mainensis* in culture *in vitro*. A: Multiplication of shoots. B: Rhizogenesis.

MATERIALS AND METHODS

Development of a complete *in vitro* conservation methodology involved multiple steps as follows: collection of plant material from natural habitats; use of adequate sterilisation procedures and aseptic *in vitro* culture initiation; characterisation of *in vitro* reactivity; optimisation of the regeneration rate; selection of medium and long-term maintenance procedures; and characterisation and evaluation of the resultant plant material.

The starting material for *in vitro* culture was *Dianthus mainensis* seeds collected from the Borus Ridge in Krasnoyarsk Krai in July 2010. Seeds were surface sterilised in a 20% Domestos solution for 20 min. The sterilised seeds were rinsed several times with autoclaved distilled water. For *in vitro* germination, 0.6% agar was used (Difco, USA).

For multiplication of shoots, the culture media was based on the Murashige and Skoog formula (1962, MS) supplemented with 6-benzylaminopurine (BAP) ranging from 0–10 μM (ICN Biomedicals, USA). For explant rooting, half strength MS basal medium supplemented with α-nephthaleneacetic acid (NAA) at 0–5 μM (AppliChem, Germany) was used. Carbohydrates were the source of sucrose (30 g/l), and the pH was adjusted to 5.8 prior to autoclaving with 1M NaOH. The duration of the process was 30–35 days. To study *in vitro* morphogenesis, the following indices were taken into account: coefficient of propagation as the number of developed shoots per explant, length of shoot (mm), number of leaves per shoot, rooting frequency (%), number of roots per regenerant, and average length of roots (mm).

Cultures were incubated at a constant temperature of 25–28C° under 16-h daily exposure to 3000 lux illumination from cool-white fluorescent lamps. Seeds were germinated under light and dark conditions.

Table. 1. Effect of concentration BAP on shoot formation of *Dianthus mainensis*, n=30.

BAP, µM	Characteristics			
	Mean number of shoots per explant	Length of shoot, mm	Number of leaf on one shoot	Vitrification, %
0	0	31.4±1.2	7.5±0.6	0
1	2.8±0.6	24.1±1.2	7.0±0.8	0
3	5.5±0.9	20.1±1.6	6.3±0.5	0
5	4.6±1.0	22.5±1.4	5.9±0.8	72

Regenerants obtained *in vitro* were adapted to unsterile conditions in the following substrates: sand: vermiculite (1:1); sand: neutral soil (1:1); carbonate soil: vermiculite (2:1); and sphagnum: vermiculite (1:1). The plants were covered with polythene film to increase air humidity in the first 5 days of adaptation.

After acclimatisation, the plants were transferred to the experimental plot "Systematicum" at the Central Siberian Botanical Garden, SB RAS (Novosibirsk). Growth observations were recorded for plants in an open field for 3 years.

RESULTS

Dianthus mainensis seeds were germinated in culture after 2 days in light and dark conditions. The germination rate was 92% when the sterility of the material was 100%.

At the multiplication stage, cytokinins are generally used with or without auxin in the basal medium to remove apical dominance and obtain the maximum number of shoots. For propagation of *Dianthus henteri* Heuff. ex Griseb. and *Dianthus nardiformis* Janka, the optimum ratio of cytokinin: auxin was shown to be 10: 1 (Cristea et al., 2010; Holobiuc et al., 2010). For *D. caryophyllus* L. and *D. superbus* L., optimal shoot development was noted in basal medium with 4 mg/l BAP (Ali et al., 2008) and 1 mg/l BAP (Mikulik, 1999), respectively. We studied the effect of the cytokinin BAP on *Dianthus mainensis* shoot formation (Table 1).

The optimal BAP concentration in this study was 3 µM (Table 1, Fig. 1A), which yielded the maximum number of shoots (5.5 shoots per explant). Concentrations of 5 µM BAP and higher are not effective due to tissue vitrification. Use of only cytokinin (BAP) in the basal medium for no more than 30–35 days ensures direct morphogenesis of *Dianthus mainensis* by activating development of pre-existing lateral meristems on a shoot. This morphogenesis preserves the genetic stability of the plant clones obtained without the appearance of somaclonal variants (Kunah, 1997).

During rhizogenesis, the mineral composition of the basal medium was a decisive influence on the development of root systems. In half strength MS media, 100% rooting of shoots was observed compared to full strength MS (Table 2, Fig. 1B).

Fig. 2. A: Flowering plants *Dianthus mainensis* in open field after 3 months of culturing. B: Adaptation of plants *Dianthus mainensis* to *ex vitro* conditions on sand: vermiculite mixture (1:1). C: Two months plant *Dianthus mainensis* in the open field.

In basal media supplemented with different concentrations of auxins, only callus formed at the base of the shoot.

Table. 2. Growth rates of *Dianthus mainensis* at the stage rhizogenesis, n=30.

Characteristics	Medium variants	
	½MC	MC
Rooted shoots, %	100	64
Length of shoot, mm	43.0±5.6	31.4±1.2
Number of leaf on one shoot	10.8±1.6	7.5±0.6
Number of roots on one shoot	7.3±1.6	6.5±1.8
Length of roots, mm	25.3±5.3	21.1±4.8
Roots of the second order, +/−	+	+

The regenerants were transferred to various substrates for adaptation. All of the substrates except for the soil mixture containing sphagnum were suitable for adaptation. This confirms the strict substrate adaptation of *Dianthus mainensis* to neutral and alkaline soils. After one month, the plants (200 samples) were transferred to a field containing $CaCO_3$ in the soil mixture (Fig. 2). The regenerants had well-developed root systems and above-ground parts, contributing to a high 83% rate of establishment in the field.

DISCUSSION

For *ex situ* conservation of *Dianthus mainensis*, an endemic plant from the West Sayan, seeds were collected from the described species location.

In vitro propagation protocols have been developed for different types of *Dianthus* (Mikulik, 1999; Paunescu and Holobiuc, 2003; Pareek et al., 2004; Ali et al., 2008; Holobiuc et al., 2010; Cristea et al., 2010) but studies regarding the *in vitro* conservation of *D. mainensis* have not been undertaken yet. The results of propagation and conservation *in vitro* of *D. mainensis* presented at the first time

The main objective of this study was to develop an efficient method for direct regeneration, which provides genetic stability of the resulting plant material. For conservation purposes, it is important to have an optimised micropropagation protocol that induces satisfactory regeneration of vigorous, rooted plants and minimises the use of high levels of growth regulators.

In general, the protocol developed meets these requirements. MS medium with cytokinin (3 μM BAP) showed good rates of regeneration without vitrification. Additional procedures for conservation of this species can be developed and included in the protocol over time.

Rooting and adaptation of plants was not difficult and could be implemented at any time. The greatest rooting percentage was obtained in half strength MS medium without growth regulators. For plant adaptation, optimal results were achieved when using sand: vermiculite (1:1), sand: neutral soil (1:1), and carbonate soil: vermiculite (2:1) mixtures. At present, *Dianthus mainensis* regenerants are being successfully cultivated in experimental fields.

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