CONSERVATION OF ENDEMIC SPECIES *DIANTHUS CALLIZONUS* SCHOTT & KOTSKY USING *IN VITRO* TECHNIQUES

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The main purpose of this research is to develop the most suitable techniques of micropropagation adapted for middle term conservation of the rare endemic species *Dianthus callizonus*. Micropropagation is a method to obtain valuable collection and allow the conservation of unique genetic resources of this species.

Key words: *Dianthus callizonus*, *in vitro* techniques, conservation.

INTRODUCTION

Endemics from the Romanian Flora are 4.87% of all superior plants (from 3 136 species [4], 153 are endemics). From these only 5.27% have a large population, but the most of the endemic taxa, 81.11%, are rare, located in restricted habitats [9]. One of those endemic rare species is *Dianthus callizonus* Schott& Kotsky (Photo1), known as the "pink of Piatra Craiului", because it is found in Piatra Craiului Massif area exclusively. This species is considered the emblem of the Meridional Carpathians and often the emblem of the Romanian Flora [5]. It is very precious from the scientific point of view – it is a relict unique taxon – but also a species with horticultural importance because it can produce natural hybrids with *D. spiculifolius* Schur (Photo 2) and *D. tenuifolius* Schur and can be used to create new valuable pink varieties.

The loss of plant genetic resources, especially wild species, has made necessary the development of "ex situ" conservation techniques to guarantee their preservation [2]. A possible method to protect this rare endemic species is multiplying by using "in vitro" cultures. This method allows conservation and also provides a valuable vegetal material for the amelioration experiments [6, 7, 14].

In Romania, the research in the field of endemics micropropagation concerns only three species: *Astragalus peterfii* [12], *Dianthus spiculifolius* [3, 13] and *Andryala levitomentosa* [1].

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MATERIAL AND METHODS

The samples were collected from Piatra Craiului Massif (Piatra Mică, 1816 m high), in flowering and fructification stages.

The explants for *in vitro* culture were foliar fragments (1 cm), defoliated stem (nodes and internodes) fragments (1.5 cm) and shoots (0.5–1 cm). The starting material was sterilized in a mercuric chloride solution (0.1 %), for 5 minutes (shoots), for 7 minutes (foliar fragments), for 10 minutes (defoliated stem fragments) and for 20 minutes (seeds) and rinsed repeatedly with sterile water. The sterile explants were incubated on a range of media (M1-M9) based on MS basal media [11], with half or full strength of mineral components. As growth regulators the variants contain different auxins as 1-naphthalene acetic acid (ANA) and 2.4 dichlorphenoxyacetic acid (2.4D) and cytokinines as 6-benzylaminopurine (BAP), Zeatin and Kinetin. As carbon source sucrose was used. A variant also contains ascorbic acid as supplement. All variants of media have a pH about 5.8 ± 0.2. The composition of media is given in Table 1.

Table 1

Composition of media

Components	units	M1	M2	M3	M4	M5	M6	M7	M8	M9
Macroelements		MS	MS	MS	MS	MS	MS	MS	½ MS	MS
Microelements		MS	MS	MS	MS	MS	MS	MS	½ MS	MS
ANA	mg/l	0.1	0.1	–	0.1	0.1	–	1	0.1	1
2.4 D	mg/l	–	–	–	–	–	1	–	–	–
BAP	mg/l	1	–	–	–	1	–	0.1	–	0.25
Kinetin	mg/l	–	1	–	–	–	0.5	–	–	–
Zeatin	mg/l	–	–	–	2	–	–	–	–	–
Sucrose	g/l	30	30	30	30	30	30	30	20	30
Ascorbic acid	mg/l	–	–	–	–	–	15	–	–	–

The cultures were maintained in a 12 h photoperiod (2 000 lux) at 25 °C.

RESULTS AND DISCUSSION

In the way to find a suitable method for micropropagation of endemic species *Dianthus callizonus*, two sources of explants were used: vegetative organs of mature plants and seedlings derived by sterile sowing.

Sterilised seeds were sown on M3 variant of media. After 30 days of culture seedlings were cut into pieces (1 cm), and placed on M1 and M2 variants of media. After 7 days of culture, foliar fragments placed on M1 variant generate callus because of the cytokinin content (BAP, 1mg/l) that stimulates cellular division. But regeneration *via* callus is not desirable because of the somaclonal variability

Photo 1 – ***Dianthus callizonus*** – original habitat.

Photo 2 – ***Dianthus*** – hybrid (*callizonus* × *spiculifolius*).

Photo 3 – Callus developing.

Photo 4 – Indirect organogenesis.

Photo 5 – Axillary shooting.

Photo 6 – Shoot developing.

Photo 7 Shoots rooting.

Photo 8 – Rooted shoots.

[8, 10, 15]. The same situation but with low intensity (after 14 days of culture) was observed when the foliar fragments were placed on M2 variant, this time because of the kinetin content (1 mg/l). After 10 days of culture nodal segments placed on M1 variant develop shoots from axillary buds (the average was 6 shoots/explant), because of the BAP content that reduces the apical dominance (Photo 6). These shoots start rooting after 17 days of culture because of the AIA content (0.1 mg/l). A similar evolution was registered at segments placed on M2 variant but with a lower rate (2 shoots/explant), because of the kinetin influence.

Cuttings from mature plants vegetative organs were placed on different medium variants. Cultures were initiated by shoots placed on M5 and M9 variants of media and by foliar and defoliated stem fragments placed on M9 variant.

After 20 days of culture shoots placed on M9 variant elongate slowly. Elongated shoots were placed on M3, M4, M5 and M7 variant of media. On M3 variant shoots start rooting after 30 days of culture, the average was 2 roots/explant (Photo 8). After 30 days of culture on M4 variant, an intense shooting process was developed from axillary buds because of zeatin influence with an average about 7.6 roots/explant (Photo 5). After 40 days of culture shoots start rooting because of the low content of auxin (ANA, 0.1 mg/l). A similar evolution was registered on M5 and M7 variants with an average of 6.2 shoots/explant (Photo 7) and 8.1 shoots/explant and after 45 days of culture start rooting.

Cultures initiated by shoots placed on M5 variant elongate slowly, after 20 days of culture. Transferred on M8 variant shoots start rooting after 15 days of culture, with an average of 2 roots/explant, because of the low auxin content (ANA, 0.1 mg/l).

Leaves and defoliated stem fragments cultured on M9 variant of media developed tissue callus after 18 days of culture (Photo 3). Callus was transferred on M4, M6 and M7 variants of media. After 30 days of culture on M4 variant callus has a significant growing rate because of the zeatin content that stimulates cellular division and callus formation. After 40 days of culture from callus arise roots with an average of 4 roots/explant and shoots, with an average of 2 shoots/explant, because of the low content of auxin (ANA 0.1 mg/l), (Photo 4). On M6 variant callus has an intense growing rate because of the auxin content (2.4D, 1 mg/l), but no organogenesis process was developed after 40 days of culture. A lower growing rate was observed on M7 variant (because of the low content of cytokines), but after 30 days of culture shoots (indirect organogenesis) arise with an average of 3 shoots/explant.

CONCLUSIONS

Regeneration of genetically stable plant can be reached if *in vitro* culture does not include the stage of callus proliferation, because callus is a source of

somaclonal variation. From this reason it seems to be more suitable for micropropagation of the endemic species *Dianthus callizonus* to choose a variant of media on which the creation of callus is not remarkable and also an appropriate starting material. A suitable way is multiplication from existing meristems such as axillary buds. In this way an efficient strategy is to cultivate shoots on M9 variant of media and after 10 days of culture to transfer the shoots on M4, M5 or M7 variant for axillary shoots developing and rooting. The most efficient micropropagation strategy is to use as starting material shoots from seedlings placed on M1 variant of media that induces axillary shoots developing which start rooting after 15 days of culture.

On the other hand, induction on somaclonal variation offers the opportunity to enhance genetic variability in plants with implications in adaptability to their natural habitats or other less exposed to risk factors. For this reason we have also analysed the regeneration *via* the callus. The best result was obtained using foliar cuttings placed on M9 variant of media for callus developing. After 20 days of culture callus was transferred with best results on M4 variant of media. In this case, different auxins stimulate distinct processes: callus proliferation (2.4 D, 1 mg/l) but also indirect organogenesis (ANA, 1 mg/l).

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