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IMPROVEMENT OF THE MICROPROPAGATION AND *IN VITRO* MEDIUM-TERM PRESERVATION OF SOME RARE *DIANTHUS* SPECIES

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Abstract: The continuous loss of plants species involves the development of the conservation strategies, the establishment of micropropagation protocols and *in vitro* collections that ought to help the *ex situ* conservation of rare and threatened plants. *In vitro* active gene bank is based on living plant tissues culture collection maintained during medium-term period of time. The aim of our work was to establish efficiently micropropagation protocols and to preserve medium term five rare *Dianthus* species from the alpine Flora: *Dianthus tenuifolius* Schur., *Dianthus callizonus* Schott et Kotschy, *Dianthus spiculifolius* Schur., *Dianthus superbus* L. ssp. *speciosus* (Reinchenb) Pawl, *Dianthus glacialis* ssp. *gelidus* Schott, Nyman et Kotschy. Good results concerning the micropropagation *and in vitro* conservation were obtained in all *Dianthus* species studied. The proliferative tissue cultures could be medium term preserved using media supplemented with different mannitol concentration.

Keywords: improved micropropagation, medium-term preservation, rare, Dianthus

Introduction

Since 1970' s, the scientists have become aware that the plant biodiversity dramatically declined. In Europe, from about 12.000 plant species, over 2000 have been considered rare or endangered (2). The legacy of the Earth Summit and the mandates of the Convention of the Biodiversity have influenced the propagation of the rare and threatened native plants.

The Strategy of *ex situ* conservation relies on the establishment of gene banks containing appropriate gene pool sampling, on the use of the micropropagation and of cryopreservation techniques and adequate strategies for reintroduction in the natural habitats of the endangered species [1, 9, 10, 11, 21].

Although Romania is rich in biodiversity (particularly the large size and quality of valuable ecosystems and the quantity of some species), in the last years being registered a progressive loss of biodiversity as a result of human activity (the agriculture, to the industrial development, intensive resources extraction and overexploitation) owing to the pollution and the climatic changes. In Romania, exist about 3,700 species of higher plants. Among them, 23 species are declared as natural monuments, 74 species are extinct, 39 are endangered, 171 are vulnerable and 1,256 are rare species [16].

A very high percent of the species of plants (4%) are endemic. In total, there are 57 endemic taxa (species and subspecies) and 171 subendemic taxa (with their territory mostly in Romania). 75% percent of the endemic and subendemic species are found in the Carpathian Mountains.

There are specified 608 taxons with different degrees of vulnerability (17.6% in The Red List) belonging to different IUCN categories [2]. It is also mentioned that 4,5% species (1189) from 3976 species and subspecies belonging to Romania Flora are endemic [8].

Taking into account the continuous loss of plants species, the protection of the habitats represents the main goal of the conservation, but the establishment of

micropropagation protocols ought to help the conservation of threatened species from unique habitats and remote locations.

The *ex situ* conservation methods are: storing germoplasm of a species from threatened habitat as seeds or in gene banks, growing living collections in botanical gardens and field cultures, *in vitro* plant tissue micropropagation and collections of the rare plants.

Over 10.000 threatened plant species are already conserved worldwide in living collections (in botanical gardens, seed banks and tissue collections). *In vitro* active gene bank is based on living plant tissues culture maintained during medium-term period of time.

Our aim was to establish and to improve *in vitro* protocols for micropropagation and medium term preservation in several *Dianthus* rare species.

Material and Methods

Five *Dianthus* rare species were studied concerning the improvement of their *in vitro* reactivity and ability to be maintained during several months or years in culture: *Dianthus tenuifolius* Schur., *D. callizonus* Schott et Kotschy, *D. spiculifolius* Schur. and *D. superbus* L. ssp. *speciosus* (Reinchenb) Pawl, *D. glacialis* ssp. *gelidus* Schott, Nyman et Kotschy (Tab. 1).

Table 1: The origin habitat of the rare *Dianthus* species

No.	The species studied	The origin habitat
1	*Dianthus tenuifolius* Schur.	M. Rarau, 1400 m altitude, Bucegi Massif, Varful cu Dor, Sinaia, 1700-1800 m altitude
2	*Dianthus callizonus* Schott et Kotschy	Piatra Craiului Massif, 1800 m altitude
3	*Dianthus spiculifolius* Schur.	Bucegi Massif, Valea cu Brazi, Sinaia, 1400 altitude
4	*Dianthus superbus* L. *speciosus* (Reinchenb)Pawl	Moroieni, 1000 m altitude
5	*Dianthus glacialis* ssp. *gelidus (*Schott, Nyman et Kotschy) Tutin.	Bucegi Massif, Valea Babei, 1800 m altitude

Some of the plant material already exist *in vitro* cultured in our laboratory from a previously experiment (as *D. callizonus, D. tenuiflolius* collected by Păunescu A. in 2003), others were collected from origin habitat and *in vitro* introduced later (*D. glacialis* ssp. *gelidus, D. tenuifolius, D. superberbus* ssp.*speciosus , D. spiculifolius* collected by Holobiuc I. in 2003, 2004, 2005).

The sterilization procedure applied for the initiation of aseptic *in vitro* cultures was represented by the washing of explants in running tap water for 1-2 hours, then the immersion of the single node stem fragments in 70° ethyl alcohol (30 seconds), the desinfection with 0.1% $HgCl_2$ (5-6 minutes), followed by three washing with sterile distilled water.

The proliferative cultures obtained in the first step through multiple axillary shooting were subsequently transferred on different media variants to enhance a high micropropagation efficiency (Tab. 2). It were tested many culture media variants having macro- and microelements according Murashige&Skoog formula [15], or reduced on half, supplemented with B_5 vitamins (12) and pH 5.8. Into culture media different growth factors and supplements have also been added. For the medium-term preservation of the *in vitro* cultures it were used three medium variants based on Murashige&Skoog formula [15] supplemented with 30 g/l sucrose and mannitol in concentrations of 3%, 6% and 9%.

Five single node stem fragments were cultured in 4 cm diameter Petri dishes, at least 10 cultures/ mannitol treatments.

The mean number of the regenerants obtained per the initial inoculum was scored after 2 months in the case of the proliferation media and after 3 months in the medium term preserved cultures.

The tissues cultures were maintained in the growth chamber, at 25°C, at 4000-lux illumination and a photoperiod of 16/8 hours.

Table 2: The composition of culture media tested for the improvement of the micropropagation efficiency and medium term preservation in *Dianthus* species

Components		**Media variants**												
		M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**
Macroelements		MS 1/2	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS
Microelements		MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS	MS
B Complex Vitamins		B5	B5	B5	B5	B5	B5	B5	B5	B5	B5	B 5	B 5	B5
C vitamin (mg/l)		30												
Growth factors (mg/l)	**BAP**	1	1	1	1	1	1	1	-	-	-	-	-	-
	Kin	-	1	1	1.5	-	-	-	-	-	-	-	-	-
	Tdz	-	-	-	-	-	-	-	0.05	0.05	0.05	-	-	
	Ad	-	-	-	-	-	100	-	-	100	-	-	-	
	NAA	0.1	0.2	-	0.1	0.1	0.1	0.1	0.01	0.01	0.01	-	-	-
	2,4- D	-	-	0.2	0.1	-	-	-	-	1	-	-	-	-
	GA_3	-	-	-	0.5	-	-	-	-	-	-	-	-	-
Different supplements (g/l)	**CA**	0.5	-	-	-	-	-	-	-	-	-	-	-	-
	Glut	-	-	-	-	0.4	-	-	0.4	-	-	-	-	-
	PVP	-	-	-	-	-	-	10	-	-	10	-	-	-
	Man	-	-	-	-	-	-	-	-	-	-	30	60	90
Sucrose (g/l)		30	30	30	30	30	30	30	30	30	30	30	30	30
Agar (g/l)		8	8	8	8	8	8	8	8	8	8	8	8	8

Abbreviations: MS- Murashige & Skoog medium, (Murashige&Skoog, 1962); B5 - Gamborg vitamins (Gamborg, 1968); BAP - benzyl aminopurine; Kin- kinetin; Tdz- thidiazuron; Ad- adenin; NAA - alfa-naftyl acetic acid; 2.4-D - 2,4 diclor fenoxy-acetic acid; GA_3 - gibberelic acid; Glut - glutamine; polyvinil-pyrrolidone, Man- mannitol, CA - active charcoal.

Table 3: *In vitro* culture response of rare *Dianthus* species

Medium variant	*D. tenuifolius*		*D. callizonus*		*D. spiculifolius*		*D. superbus* ssp.*speciosus*		*Dianthus glacialis* ssp. *gelidus*	
	a	***b***	***a***	***b***	***a***	***b***	***a***	***b***	***a***	***b***
M1	20-40	**30**	20-30	**26.6**	20-40	**31.6**	20-40	**30**	15-20	**12.5**
M2	20-50	**37.2**	20-50	**33.3**	30-50	**37**	30-50	**36.6**	10-20	**15**
M3	30-50	**46**	20-50	**36**	30-50	**40**	30-50	**36.6**	15-25	**15.6**
M4	50-100	**81**	40-50	**43**	50-100	**83.3**	50-100	**75**	40-50	**43.3**
M5	15-20	**18**	10-20	**15**	20-30	**23.3**	-	**-**	7-9	**7.6**
M6	15-25	**21.6**	15-20	**17**	30-40	**33.3**	-	**-**	10-15	**11.6**
M7	10-25	**15**	10-25	**15**	20-40	**30**	-	**-**	7-15	**10.6**
M8	15-20	**17**	6-15	**12**	20-30	**25**	-	**-**	6-15	**10.3**
M9	20-30	**25**	15-20	**18**	20-40	**25**	-	**-**	10-20	**15**
M10	15-20	**15**	10-15	**12.3**	20-30	**26.6**	-	**-**	7-15	**12**
M11	20-30	**25**	20-30	**26.6**	20-30	**25**	20-30	**25**	15-20	**14.3**
M12	60-80	**71.6**	50-100	**75**	50-100	**83.3**	50-70	**63.3**	40-50	**45**
M13	40-50	**45**	30-70	**50**	50-70	**54**	40-50	**45**	40-60	**50**

Legend: a - Minimum and maximum number of registered regenerants /inoculum; b - The mean number of regenerants/ inoculum

Results and Discussions

In Romania, several authors considered that it is necessary to improve the micropropagation and conservation methods in the species with high degree of vulnerability [4, 5, 23]. The establishment of micropropagation protocols for phytoendemits would allow their introduction in the conservation and in the restoration programs [11]. Until now, there were performed several studies concerning *in vitro* culture in some *Caryophyllaceae* rare species [3, 6, 7, 17, 18, 22] but protocols for medium term preservation of the proliferatives cultures were not reported.

The mean regeneration rates scored after the first culture and after the second transfer varied according to the species and the medium, but in all species tested were high (Tab. 3). In all *Dianthus* rare species, the micropropagation rate was improved and *in vitro* tissues cultures could be maintained in good condition during several months in a collection of rare plants.

In all the cases, the media with cytokinin dominance allowed the multiple axillary shoots induction in the first step, and then the regeneration continued with the direct morphogenesis (shoots formation).

Dianthus tenuifolius was responsive on all media tested. Medium M1 (MS salts reduced on half supplemented with ascorbic acid and active charcoal) allowed a regeneration rate of 30-shoots/ inoculum. The use of M2 and M3 media (with BAP and kinetin associated to NAA or 2.4-D), allowed a high regeneration rate through direct morphogenesis (with a maximum of 50 shoots/explant).

The variants supplemented with thidiazuron were not proven to be more efficiently comparing to the classic proliferation media. The adding of different supplements (glutamine, PVP) did not influence significantly the regeneration rate. The presence of adenine in M6 media had a beneficial effect, but associated to thidiazuron (in M10 variant) determined a good regeneration rate with a vitrification tendency.

Dianthus callizonus had also a good *in vitro* behaviour, being studied in previous works [17, 18], but the reported regeneration rate did not exceed 8 shoots/inoculum. In our study, the best multiplication rate was improved, being scored on M4 variant (about 50 shoots/ explant). The media supplemented with thidiazuron, although induced a good regeneration, the number of shoots could not exceed those obtained on the M1-M4 variants.

D. spiculifolius and *D. superbus* ssp. *speciosus* had a similarly *in vitro* responses. Both species were characterised by high regeneration response and increased growth rate (Fig.1c). There are some previous works concerning the micropropagation in *D. spiculifolius* [3,6].

Our results are accordingly to those reported by V. Cristea and al. [6] in a previously study. The subculture of the neo-formed shoots on M2, M3 and M4 variants determined the significant increase of the *in vitro* reactivity (about 50-100 shoots/ explant). Mikulik [14] performed some studies concerning micropropagation in *D. superbus*, the best results of axillary shoot induction were obtained on medium supplemented with BAP, but the regeneration rate was lower than we have obtained.

Dianthus glacialis ssp. *gelidus* was *in vitro* cultured starting from single node stem fragments, the mean regeneration rate varying between 7.6 and 43.3 shoots (Tab. 3). In all variants, plantlets were regenerated in the first step through multiple shooting from the secondary buds and then through direct morphogenesis (Figs. 1 a,b).

All the *Dianthus* species studied had a high multiplication rates and an increased elongation rate of the shoots even on control hormene -free medium in a short time interval. For these resons, for medium term preservation, it is strongly imposed the establishment of protocols for the reduction of the number of the transfers and of the growth rate.

The conservation using *in vitro* tissues cultures generally relies on the control of the growth [4]. The short and medium term conservation of the plant germplasm can be achieved through the reduction of the growth using different methods (through diminishing of the mineral

Fig. 1: a -Direct morphogenesis in *Dianthus glacialis* ssp. *gelidus*; b - Regenerated plantlets of *D. glacialis* ssp. *gelidus;* c - *Dianthus spiculifolius* shoots after 3 weeks of culture on control medium; d - Medium term miniature *in vitro* culture of *D. callizonus* maintained during 12 months on 3% mannitol supplemented medium; e, f - Somatic embriogenesis in *D. spiculifolius* and *D. tenuifolius* cultured 3 months on 9% mannitol supplemented medium.

nutrients content, of the carbon source, the use of a medium stress factor, reduction of the temperature, of the light). The successfully implementation of the minimal growth technologies involves the elaboration of specific protocols for every species.

In our study, we used three mannitol concentrations for the reduction of growth. In the tissue culture, mannitol changes the osmotic potential of the cells acting as osmolyte. Mannitol is a sugar alcohol, already have been reported its antioxidant role in plants [19].

The media variants supplemented with the mannitol in concentration of 3% determined the reduction of the growth rate and limitation at 1-2 nodes of the plantlets and a satisfactory regeneration rate even in absence of the growth factors. These shoots did not grow; they remained at single or two node stage as mini plants (Fig. 1d). This plant material *in vitro* cultured can any time transferred on appropriate medium to growth and to form normal developed plants. This protocol is adequate for the preservation of *in vitro* tissues cultures collection in a very reduced space and without any supplementary handling.

The addition of mannitol at concentration of 6% determined the maintenance of shoots at single node stage and the stimulation of the regeneration rate, for instant through direct morphogenesis, and subsequently during the time (along 3-4 months) the induction of the somatic embryogenesis.

The level of 9% mannitol, during 1 month after the inoculation of the single node cuttings, induced an inhibition of regeneration and growth of the inoculum. After 2-3 months of culture, this level of osmotic stress acted subsequently as a switcher of the regeneration, inducing somatic embryogenesis process with increased rate (~100 regenerants/ initial inoculum) (Figs.1e,f). In a 4 cm diameter Petri dishes, are produced hundreds of embryos or miniplants. It is already reported that other osmotic stress factor like polyethylene glycol can enhance embryo formation [13]. The mannitol induces, even in absence of the growth factors the high stimulation of the regeneration, in the meantime, the regenerants being maintained at 1-2 mm high, as embryos or very small plants. The proliferative cultures don't need transfers and large culture vessels. In this way could be obtained medium-term preserved miniature cultures with extremely high regeneration rate.

Conclusions

- The species tested had a good *in vitro* behaviour, being suitable for the establishment of an active *in vitro* gene bank.
- In all *Dianthus* rare species were elaborated improved micropropagation methods**.**
- The proliferative tissue cultures can be medium term preserved using different mannitol concentration. The method is adequate for the preservation of *in vitro* tissues cultures collection in a very reduced space and without any supplementary transfers.
- The presence of mannitol in concentration of 6% and 9% stimulates the direct embryogenesis process. This plant material could be used for producing of the synthetic seeds or for long storage (cryopreservation procedures).

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OPTIMIZAREA MICROPROPAGĂRII ŞI CONSERVAREA PE TERMEN MEDIU A UNOR SPECII RARE DE *DIANTHUS*

(Rezumat)

Diminuarea continuă a numărului de specii de plante impune dezvoltarea unor strategii de conservare. Stabilirea de protocoale de micropropagare şi de colectii *in vitro* trebuie să faciliteze conservarea *ex situ* a plantelor rare şi periclitate. O bancă activă de gene se bazează pe culturi vii de țesuturi menținute pe termen mediu.

IN VITRO MICROPROPAGATION AND MEDIUM TERM PRESERVATION OF SOME RARE *DIANTHUS* SPECIES

Scopul lucrării noastre a fost stabilirea şi optimizarea unor protocoale de micropropagare şi conservarea pe termen mediu a unor specii rare de *Dianthus* din flora alpină: *Dianthus tenuifolius* Schur., *D. callizonus* Schott et Kotschy, *D. spiculifolius* Schur., *D. superbus* L. ssp. *speciosus* (Reinchenb) Pawl şi *D. glacialis* ssp. *gelidus* Schott, Nyman et Kotschy. Toate speciile studiate au avut o bună reactivitate în cultura *in vitro*. Culturile proliferative au putut fi conservate pe termen mediu cu succes utilizând medii adiționate cu diferite concentrații de manitol.