Contribuţii Botanice, XLI, (2), **2006**
Grădina Botanică "Alexandru Borza"
Cluj-Napoca

MICROPROPAGATION OF *DIANTHUS PYRENAICUS* POURR. - ENDEMIC SPECIES FROM PYRENEAN MOUNTAINS

***Delia MARCU*[1], *Victoria CRISTEA*[2], *Anca BUTIUC KEUL*[3]**
[1] Universitatea "Babeş-Bolyai", Facultatea de Biologie şi Geologie, str. Republicii, nr. 46, **RO-40015 Cluj-Napoca**
[2] Universitatea ,,Babeş-Bolyai", Grădina Botanică ,, Al. Borza", str. Republicii, nr. 42, **RO-40015 Cluj-Napoca**
[3] Institutul de Cercetări Biologice, str. Republicii, nr. 48, **RO-40015 Cluj-Napoca**
e-mail: victoriacristea@grbot.ubbcluj.ro

Abstract: The biotechnology of *in vitro* culture is increasingly used as part of *ex situ* and *in situ* programmes for the conservation of a number of plant species that are endemic, endangered, vulnerable or rare. *Dianthus pyrenaicus* Pourr. is mentioned in Flora Europaea as en endemism for the French south part and the Spanish north-east part of the Pyrenean Mountains [1, 9]. The aseptic culture was induced starting from sterile seeds. The culture were induced and then maintained on aseptic media, with a varied hormone balance in order to obtain an as high possible multiplication rate and induction of rhizogenesis. After the acclimatization the plants obtained from this experiment will be cultivated in the Botanical Garden ,,Alexandru Borza", from Cluj-Napoca, in the area of the Alps, Apennines and Pyrenees Mountains flora. The initiation of the culture and the multiplication of *Dianthus pyrenaicus* Pourr. using the *in vitro* biotechnology has been leading to a much better results than the normal culture in the greenhouse.

Keywords: *Dianthus pyrenaicus* Pourr., *in vitro* culture, *ex situ* conservation, endemic species, infection index, phytohormone balance, multiplication ratio, rhyzogenesis, *ex vitro* acclimatization.

Introduction

Conservation of numerous species of the spontaneous flora that are endemic, endangered, vulnerable or rare is being realised by *in situ* or *ex situ* methods. The biotechnology of *in vitro* cultures is an important part of these programs. *Dianthus pyrenaicus* Pourr. is an endemic species for the Pyrenees Mountains, found in the south French and in the north-east Spanish part [1, 9]. These mountains ranges, the largest in Spain, are rich in species, *e.g.* it can be found here 3500 species of plants, almost half of the Iberian flora. 4 % of the plants of these mountains are endemic [7]. *Dianthus pyrenaicus* Pourr. grows on the alpine rocks and prefers the sunny places. It is a laxly caespitose perennial plant, witch can grow up to 15-45 cm, usually with long, trailing, rather slender, woody stems. The leaves are usually rigid, pungent, not densely crowded on flowering stems. Inflorescence is usually branched. Petal-limb has 3-8 mm; it is glabrous, dentate, and pink. Flowers appear on late spring – early summer. Other synonymous names of this species in Flora Europaea are *Dianthus attenuatus* Sm., *Dianthus requienii* sensu Willk. pro parte, non Gren. & Godr. and *Dianthus maritimus* Rouy. [9].

Our researches focused on the *in vitro* conservation of endemic or endangered taxa, because unfortunately, like as in the major part of the mountains, due to the excessive anthropization, the landscape diversity has decreased. These micropropagated plants are cultivated in the different area of "Alexandru Borza" Botanical Garden, from Cluj-Napoca. One of our actual goals is to introduce continuously new specific taxa in the phytogeographical sector and, for that purpose, in the Botanical Garden and in the Biological Research Institute experiments concerning the conservation of various endemic taxa through aseptic culture are being realised [2, 3, 4, 5, 8, 10].

We choose to study *Dianthus pyrenaicus* Pourr. also because we didn't found in the literature no results or experiment of inducing *in vitro* culture and conservation of this species.

Material and Methods

The aseptic culture was induced starting from plants seeds obtained from Yagellow Botanical Garden of Krakow (Poland). These were obtained as a result of the seeds exchange of the Botanical Garden ,,Alexandru Borza" of Cluj-Napoca with almost 500 similar institutions of all over the word.

The seeds sterilization where made with oxygenated water (H_2O_2). For presterilization the seeds were kept 12 hours in 4% H_2O_2 before starting the experiment. Then the vegetal material was kept for 1 minute in ethylic alcohol and then sterilized 17-18 minutes in 10% H_2O_2. After that the seeds were rinsed in sterile water.

The culture media utilized for the introduction of the vitrocultures and for the micropropagation are presented in table 1 and table 2. From the plantlets generated by the seeds germination, were used apical and nodal plant fragments.

The medium used contained different hormones (cytokinins/auxins) according to our purpose. We tried to set an optimum phytohormone balance that may allow the micropropagation and acclimatization of this species.

Table 1: The base composition of the culture media used for the micropropagatin of *Dianthus pyrenaicus* Pourr.

	Composition		Quantity/ 1l of medim
Base medium	Components according to Murashige-Skoog (1962) [6]	macroelements	100 ml
		microelements	1 ml
		FeEDTA	5 ml
	Vitamine	thiamine	1 mg
		pyridoxine	1 mg
		nicotinic acid	1 mg
	myo-inozitol		100 mg
	saccharose		20 g
	agar		7 g

Table 2: Variants of culture media used for the micropropagation of *Dianthus pyrenaicus* Pourr. depending on the phytohormones contained.

Variants	Phytohormones (mg/l)						Phytohormones balance (cytokinins/auxins)
	Auxins		Cytokinins		Giberelins	Activated charcoal	
	BAP	K	NAA	IAA	GA_3		
Media with GA_3	-	-	-	-	100	-	-
Media without GA_3	-	-	-	-	-	-	-
KN	-	1	1	-	-	-	1/1
Kn	-	1	0.1	-	-	-	10/1
KN 0.5	-	1	0.5	-	-	-	2/1
BN 0.5	1	-	0.5	-	-	-	2/1
BI	1	-	-	1	-	-	1/1
BN	1	-	1	-	-	-	1/1
C	-	-	-	-	-	1	-

The phytohormones used were:

- as cytokinins (known as stimulators for cell multiplication and for new plants forming): BAP (6-benzylaminopurine) and K (kinetin);
- as auxins (known as stimulators for the rhizogenesis): NAA (naftaleneacetic acid) and IAA (indolilacetic acid);
- and as gibberellins was used GA_3 (gibberellic acid), that is usually used to take of the seed from dormancy.

The microclimatic conditions from the vegetation room were: a temperature of 25 ± 2^0C, a light intensity of 87 µmol/m^2/s and a photoperiod of 16 h light/8 h dark.

Results and Discussion

After 25 days from inoculation of plant seeds the average infection index was low, 3.6%, and the average regeneration rate was 77.5%. The results are satisfactory, pointing out that the seeds of this species (comparing with other species) are well sterilised for induction of *in vitro* cultures. The infection ratio was higher at dark than in the light, where any seed was infected (figure1). Studying the *in vitro* culture induction for other species, *e.g. Aquilegia nigricans ssp. subscaposa* the infection ratio was higher in light than in dark (65% in light and 55% in dark) [3].

We determinate the germination ratio under GA_3 influence, both to light and dark. At dark, on the medium with GA_3, germination ratio is higher (100%) then to the light (50%). Also it can be seen that GA_3 stimulates the germination ratio in the dark (100%) - comparatively with the medium without GA_3 (70%) while in the light, this phytohormone is inhibiting it - 50% germinated seed on the GA_3 medium respectively 90% on the medium without GA_3. So, it can be said for this species that adding GA_3 to the media it's not necessary, because the germination ratio is satisfactory on the media without GA_3, too.

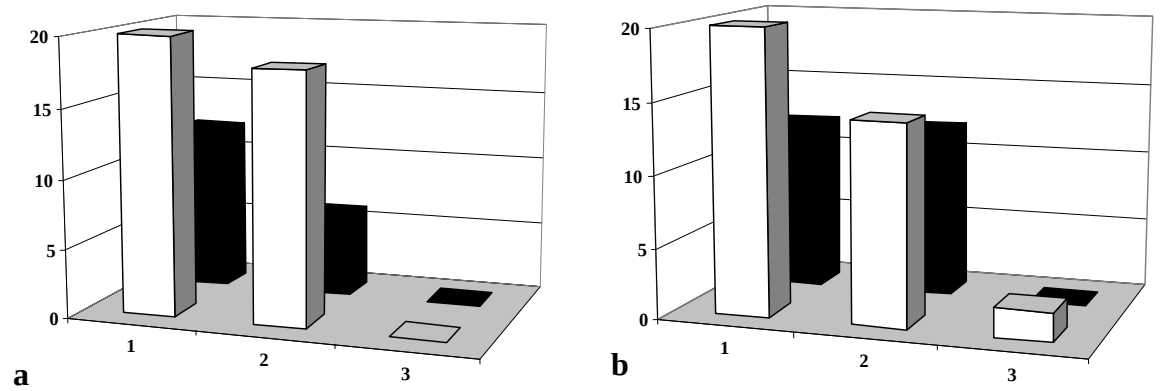

Fig. 1: Seeds germination, after 25 days from inoculation: a: in light; b: in dark. 1 = number of inoculated seeds; 2 = number of germinated seeds; 3 = number of infected seeds; □media without GA_3; ■media with GA_3.

The sterilization method, with H_2O_2, was successfully used in other experiments, for other species, when the classical sterilization with chlorine hypochlorite (like Domestos -5% NaHCl) failed [3, 5].

The results obtained at 46 days after the inoculation of sterilized seeds are shown in table 3.

Table 3: Plantlets development after 46 days from disinfection and inoculation of seeds.

	Media without GA_3			**Media with GA_3**		
	Long plants (20-40mm)	**Short plants (<20 mm)**	**Necrosated plants**	**Long plants (20-40 mm)**	**Short plants (<20 mm)**	**Necrosated plants**
%	85.2	7.4	7.4	50	10	40

As it can be seen in table 85.3% of the plantlets on the media without GA_3 and 50% of the one on the media containing GA_3 present a good evolution and a good growth. GA_3 it is known from the literature as a hormone which stimulates the growth of internodes, but in this faze of our experiment this effect of GA_3 it can't be seen.

The next experiment was realised to compare the influence of different concentration of the auxin NAA. The results obtained at 51 days from inoculation show that the low concentration of NAA is more favourable for rhizogenesis – 87.5% of plants present the phenomenon - than the higher one – 63.6%. Later on, at 81 days, we analysed the influence of the two different concentrations over the multiplication and rhizogenesis ratio. So, it can be seen that for total length/plant, ramification length/plant, number of roots/plant and length of roots/plant the low concentration of NAA is more favourable that the higher one. But for the ramification of plants the higher concentration is more favourable.

Next it was studied the influence of two cytochinins - BAP and K - over the multiplication ratio

Table 4: Multiplication ratio on media containing two different cytokinins, 70 days after inoculation.

Inocula type	Media (mg/l)	New plant forming / culture vessel	Multiplica-tion ratio	Medium ramification number/culture vessel	Medium ramification number/ino-cula	Medium new plant length/ inocula (cm)	New plant length/ culture vessel (cm)
Apical	NAA=0.5; BAP=1	123	33.9	13.4	4.1	88.7	325.8
	NAA=0.5; K=1	41	10.3	3	0.8	43.4	173.5
Nodal	NAA=0.5; BAP=1	115	28.8	6.7	1.7	87.3	349.4
	NAA=0.5; K=1	36.3	9.1	5.2	1.3	50	200

and the rhizogenesis (table 4, 5). The experiment was made using apical and nodal inocula. The results obtained at 70 days from inoculation are shown in table 4. In this table are also presented the results obtained/culture vessel (Erlenmeyer 100 ml) for appreciate the rateability on this kind of culture. BAP had a better influence on the multiplication for the apical inocula as well as for the nodal one, comparing with K. But f or rhizogenesis K has a better influence than BAP (table 5). A much better rhizogenesis on the media with K was also obtained for other species (*Dianthus spiculifolius* Schur) as well [4].

Comparing the influence of two different auxins (NAA and IAA) over rhizogenesis, it results that, at 109 days from inoculation, rhizogenesis is better on the media containing IAA (73.2%) than on the one containing NAA (11%). So in the case of this species, IAA is more favourable for rhizogenesis than NAA but the multiplication is favourized by NAA (Fig. 2).

Before *ex vitro* acclimatization the plantlets were transferred on two different media. One of the media contained activated charcoal (which is usually used for rhizogenesis induction), 25 g/l, without phytohormones and the other contained phytohormones (kinetin 1mg/l and NAA 2 mg/l). For this species the rhizogenesis was better on the media with phytohormones than the on with activated charcoal.

Before *ex vitro* acclimatization the plantlets were transferred on two different media. One of the media contained activated charcoal (which is usually used for rhizogenesis induction), 25 g/l, without phytohormones and the other contained phytohormones (kinetin 1mg/l and NAA 2 mg/l). For this species the rhizogenesis was better on the media with phytohormones than the on with activated charcoal.

Next for acclimatization were used the plantlets from the media containing phytohormones. These were transferred on a mixture of peat and sand or peat and perlite (Fig. 3). Until this moment the plants have a good evolution. This acclimatized plants will be planted in

the Alps, Apennines and Pyrenees Mountain flora sector in the Botanical Garden „Alexandru Borza", Cluj-Napoca.

Table 5: Rhizogenesis on media containing two different cytokinins at 70 days from inoculation.

Inocula type	Media	Root number/culture vessel	Medium length/culture vessel (cm)
Apical	NAA=0.5; BAP=1	7.00	2.25
	NAA=0.5; K=1	15	3.38
Nodal	NAA=0.5; BAP=1	2.00	0.6
	NAA=0.5; K=1	15	7.10

Media with IAA **Media with NAA**

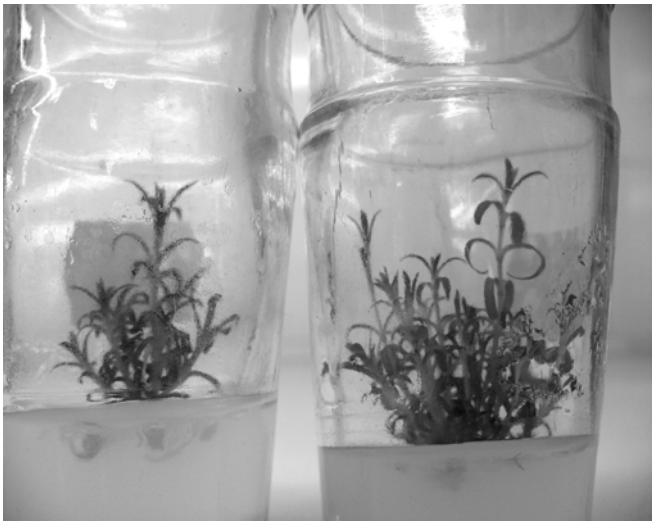

Fig. 2: Micropropagation on two different media after 109 days from inoculation

Fig. 3: The acclimatization of in vitro micropropagated *Dianthus pyrenaicus* Pourr. plantlets.

In parallel with this experiment of *in vitro* culture it was realised another one. The seeds of this species were cultivated in the botanical garden green house. The micropropagation of this species by *in vitro* cultures is more favourable than the classical one (culture in soil in the greenhouse). So the germination ratio was much higher and it was realised in a shorter period of time. This happened probably, because of the more constant and optimal condition during *the in vitro* culture, comparatively with the classical one.

Conclusion.

The studies performed resulted that concerning starting the aseptic culture of this species the sterilization with oxygenated water was satisfactory, the infection ratio was very low (3.1%). At dark the germination ratio is higher than to the light and GA_3 stimulates the germination at dark and is inhibiting it at light.

For the multiplication ratio our study revealed that the media with cytokinin BAP is more favourable than the one with kinetin. For the media with auxins, the multiplication is better on the media with NAA than on the media with IAA. We also observed tat the regenerative capacity of the apical explants is higher than of the nodal one.

For the rhizogenesis the media containing IAA was better than the one containing NAA. Also the smaller quantity of NAA (0.1 mg/l) was more favourable for rhyzogenesis than the one with a higher quantity of this phytohormones (1mg/l).

REFERENCES

1. Bernal, M., Laínz, M., Muñoz Garmendia, F., 1990, *Dianthus* L. In: Castroviejo, S., Laínz, M., López González, G., Montserrat, P., Muñoz Garmendia, F., Paiva, J., Villar, L., (eds.), *Flora Iberica II*, Real Jardín Botánico, C.S.I.C., Madrid: 426-462.
2. Butiuc-Keul, A.L., 2000, *Cercetări privind organogeneza in vitro la unele plante rare*, Teză de doctorat, Univ. „Babeş-Bolyai", Cluj-Napoca.
3. Cristea, V., Miclăuş, M., Deliu, C., Halmágyi, A., 2004, The micropropagation of some endemic and rare taxa from Gilău – M-tele Mare Massif, *Contrib. Bot.*, **XXXIX**: 201-209.
4. Cristea, V., Miclăuş, M., Puşcaş, M., Deliu, C., 2002, Influence of hormone balance and in vitro photoautotrophy on *Dianthus spiculifolius* Schur micropropagation, *Contrib. Bot.*, **XXXVII**: 145-153.
5. Miclăuş, M., Cristea, V., Deliu, C., 2003, Micropropagation on *Dianthus petraeus* W. et K. ssp. *simonkaianus* (Péterfi) Tutin, *Contrib. Bot.*, **XXXVIII**: 77-84.
6. Murashige, T., Skoog, F., 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant*., 18: 473-497.
7. Martínez-Rica, J.P., 1998, Higher plant and vertebrate species richness in Spanish and some Mediterranean mountains, *Pirineos*, **151-152**: 101-110.
8. Şuteu, A., 2002, *Cercetări asupra biologiei speciei endemice Astragalus péterfi Jáv*., Teză de doctorat, Univ. „Babeş-Bolyai", Cluj-Napoca.
9. Tutin, T.G., 1964, *Dianthus* L. In: Tutin T.G., Heywood, V.H., Burges, N.A., Valentine, D.H., Walters S.M., Webb D.A., (eds.), *Flora Europaea, I*, Cambridge University Press, Cambridge: 188-204.
10. Zăpârţan, M., 2001, *Conservarea florei spontane prin înmulţire in vitro*, Ed. ALC MEDIA GROUP, Cluj-Napoca.

MICROPROPAGAREA LA *DIANTHUS PYRENAICUS* POURR. - SPECIE ENDEMICĂ PENTRU MUNŢII PIRINEI

(Rezumat)

Munţii Pirinei sunt foarte bogaţi în specii endemice ale florei şi faunei. Astfel, din cele aproximativ 3500 specii de plante vasculare ale acestor munţi, 4% sunt endemice [9]. Una dintre aceste endemite este şi *Dianthus pyrenaicus* Pourr. citată în Flora Europaea şi în Flora Iberica ca specie endemică pentru sudul Franţei şi nord-estul Spaniei [1, 11]. Studiile noastre au urmărit inducerea culturilor *in vitro* - cu scopul conservării *ex situ* - şi micropropagarea (multiplicarea şi rizogeneza) cu un randament cât mai bun a acestei specii. Iniţierea culturii s-a

realizat pornind de la semințe sterilizate cu H_2O_2 10%, pe mediu cu, sau fără acid giberelic (GA_3), la lumină sau întuneric. După 25 zile de la inoculare rata de infecție a fost redusă, de 3,6%, iar rata de germinație a fost foarte bună, de 100%. În paralel s-a urmărit şi germinația semințelor *ex vitro* în condiții de seră, însă aceasta a fost foarte redusă. Micropropagarea - adică (1) rata de multiplicare; (2) lungimea totală a neoplantulelor/inocul; (3) numărul ramificațiilor formate/neoplantulă; (4) numărul de neoplantule/vas de cultură - este mai bună pe mediile de cultură ce conțin ca citochină benzilaminopurina (BAP) - (1) de 33,9; (2) 88,7; (3) de 4,1; (4) de 123 - în comparație cu mediile conținând chinetină (K), unde valorile sunt de (1) de 10,3; (2) de 43,4; (3) de 0,75; (4) de 41 respectiv. În ceea ce privește influența auxinelor asupra rizogenezei acidul indolilacetic (IAA) este mai favorabil (rizogeneza prezentă la 73,2% din inoculi) față de acidul naftilacetic (NAA) (unde rizogeneza apare doar la 11% din explante). NAA în schimb favorizează mai mult multiplicarea, în comparație cu IAA. Vitroplantele urmează a fi aclimatizate în sectorul Munților Alpi, Apenini şi Pirinei, din Grădina Botanică „Alexandru Borza" din Cluj-Napoca. Multiplicarea acestei specii prin culturi aseptice a condus la rezultate mult mai bune decât în cazul culturii ei clasice în sol, în seră.