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MICROPROPAGATION ON *DIANTHUS PETRAEUS* W. ET K. SSP. *SIMONKAIANUS* (PÉTERFI) TUTIN

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Abstract: The biotechnology of *in vitro* cultures represents an efficient way of multiplying plant subspecies that are rare, vulnerable or endangered. *Dianthus petraeus* W. et K. ssp. *simonkaianus* (Péterfi) Tutin grows on the calcareous rocks of the mountain belt inside Romania as well as in Macedonia, Bulgaria and Greece [1,3,8]. This subspecies' populations from our country are more and more affected by lime quarries, excessive pasturing and because the plant is harvested as an ornament. Thus, the subspecies is mentioned in The Red Lists elaborated for Romanian flora as rare [6,7,8]. For the micropropagation of this subspecies we used plant material harvested in 2001 and 2002 from Gilău-Muntele Mare Massif. The inocula used for the initiation of the aseptic cultures consisted of apical, uninodal or plurinodal explants, floral stems nods, buds and seeds. The easiest way to induce the cultures has proven to be the use of apical and nodal (uni- or plurinodal) explants, while the buds did not regenerate. The main purpose for the cultures generated from seeds was to see the influence had by the solar exposition (i.e., the exposition had by the individuals from which the seeds were harvested) upon germination and further development of the vitroplants. The results showed that northern exposition is obviously unfavoured in comparison to southern and western expositions. The medium culture used was a basic medium with several variants, depending on the phytohormones added. The phytohormones used were: NAA (naftaleneacetic acid), IBA (indolylbutiric acid) and IAA (indolylacetic acid), as auxins and as cytokinins – BAP (6 – benzylaminopurine), kinetine (6 – furfurylaminopurine), 2iP (6 – dimetilaminopurine) and TDZ (tidiazurone). By changing the hormone balance (cytokinins/auxins ratio) we had in view the achievement of a high multiplication rate along with rhizogenesis induction. On the medium containing BAP (1 mg/l) and IAA (1 mg/l) a multiplication rate of 110 neoplantlets/apical inoculum was obtained. The rhizogenesis was greatly stimulated by a ½ Murashige-Skoog medium, containing vegetal carbon (3 g/l), good results being also achieved on the media with 2iP or TDZ. The vitroplants having a well-developed root system were transferred in *ex vitro* conditions using a sterilized mixture of soil-perlit-peat. Thus, their acclimatization was achieved. For a succesfull acclimatization special caps were used for the culture recipients, equipped with a filter (suncap closure) having a porosity of 0.02 μm. The special caps allow CO_2, from the environment, to enter the recipient. Thus, the vitroplants, which are photoheterotrophic or photomixotrofic in a normal culture, are allowed to photosintetise and the shock suffered by them when they are transferred in *ex vitro* condition is significaly diminished [5].

Introduction

The biotechnology of *in vitro* cultures represents an efficient way of multiplying plant species that are rare, vulnerable or endangered.

Dianthus petraeus W. et K. ssp. *simonkaianus* (Péterfi) Tutin grows on the calcareous rocks of the mountain belt inside Romania as well as in Macedonia, Bulgaria and Greece [1,3,8]. It is a perennial, caespitose plant, with 10-30 cm high floral stems, simple or branched, bearing 1-6 white flowers, which appear in June-July. The populations from our country are more and more affected by excessive pasturing, lime quarries and because the plant is harvested as an ornament. Thus, the subsubspecies is mentioned in The Red Lists elaborated for Romanian flora as rare [6,7,8].

Although *in vitro* multiplication was done for other taxons of *Dianthus* genra [2,4,9,10], in the case of this subspecies no study has been done before (as the studied literature shows).

This paper aims to achieve a protocol of aseptic culture, which will allow a high rate of multiplication and the establishment of an optimum phytohormone balance (cytokinins/auxins)

that will assure a good micropropagation and acclimatization. We also studied the influence of the ground exposition from where the seeds were harvested upon the *in vitro* evolution.

Material and Methods

For the micropropagation of this subspecies we used plant material harvested in 2001 and 2002 from Gilău-Muntele Mare Massif. The inocula needed for the initiation of the aseptic cultures consisted of apical, uninodal or plurinodal explants, floral stems nods, buds and seeds.

The basic composition of the culture media is listed in table 1 and the variants, depending on the phytohormones contained, are listed in table 2.

Table 1: The base composition of the culture media used for the micropropagation in *Dianthus petraeus ssp. simonkaianus*.

	Composition		Quantity/ 1l of medium
Base medium	Components according to Murashige-Skoog (1962)	macroelements	100 ml
		microelements	1 ml
		FeEDTA	5 ml
	Vitamines	thiamine	1 mg
		pyridoxine	1 mg
		nicotinic acid	1 mg
	myo-inozitol		100 mg
	Saccharose		20 g
	Agar		7 g

The phytohormones used were NAA (naftaleneacetic acid), IBA (indolilbutiric acid) and IAA (indolil acetic acid) as auxins (which are known to be stimulators for the rhizogenesis) and as cytokinins (which are known to be stimulators for cellular multiplication and plantlets neoformation): BAP (6-benzylaminopurine), K (kinetine = 6-furfurylaminopurine), 2iP (6-dimetilaminopurine) and TDZ (tidiazurone).

Table 2: Variants of culture media used for the micropropagation in *Dianthus petraeus ssp. simonkaianus* depending on the phytohormones contained.

Variants	Phytohormones (mg/l)							Phytohormones balance (cytokinins/auxins)
	Auxins			Cytokinins				
	NAA	IAA	IBA	BAP	K	2iP	TDZ	
V1	0.1	-	-	1	-	-	-	10/1
V2	1	-	-	1	-	-	-	1/1
V3	1	-	-	-	1	-	-	1/1
V4	-	1	-	1	-	-	-	1/1
V5	0.5	-	-	-	-	2	-	4/1
V6	-	-	0.5	-	-	-	1	2/1
V7	0.25	-	-	-	0.25	-	-	1/1
V8	1	-	-	2.5	-	-	-	2.5/1
V9	1	-	-	5	-	-	-	5/1
V10	0.5	-	-	-	0.5	-	-	1/1
V11	0.05	-	-	0.5	-	-	-	10/1

The sterilization of plant material harvested in the field was done with Domestos 90-100%, for 15 minutes. The sterilization of seeds was done with Domestos 100%, for 30 minutes.

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

Results and Discussions

The explants originating from **vegetal material harvested in the field** were mostly apices and floral stems nods. Buds were also used but they did not regenerate. The infection index, at one week after inoculation, was 16%. This result can be considered satisfactory taking into consideration the results obtained in other species. The initial solid media were based on a Murashige-Skoog medium with NAA and K, 0.25 mg/l each (V7 medium) or 1 mg/l each (V3 medium).

At 41 days after the inoculation, the results showed that the plantlets generated from apices or nods were well developed but the callusogenesis phenomenon, at the base of the inocula, was frequent. The photosinthesis phenomenon is favored by the high concentration of phytohormones in the medium, as it is shown by the very intense green colour of the neoplantlets. The multiplication phenomenon was present on both media, for the inocula represented by apices as well as those represented by nods. In this phase of our study we obtained an average of 1-2 neoplantlets/inoculum but sometimes 4 neoplantlets/inoculum have been generated.

Because of the low multiplication rate and because the rhizogenesis phenomenon was not satisfactory, explants from the well grown inocula, having the greatest multiplication rate, were transferred on 3 different media having the following hormone balance: NAA 0.1 mg/l and BAP 1 mg/l (V1 medium), NAA 1mg/l and BAP 2.5 mg/l (V8 medium) and NAA 1 mg/l and BAP 5 mg/l (V9 medium). In the case of the first medium the evolution was mostly towards callus formation but a certain multiplication was also obtained (Fig. 1). For the other two media the inocula regenerated neoplantlets. The rate of multiplication for the media used was 4-7 neoplantlets/inoculum.

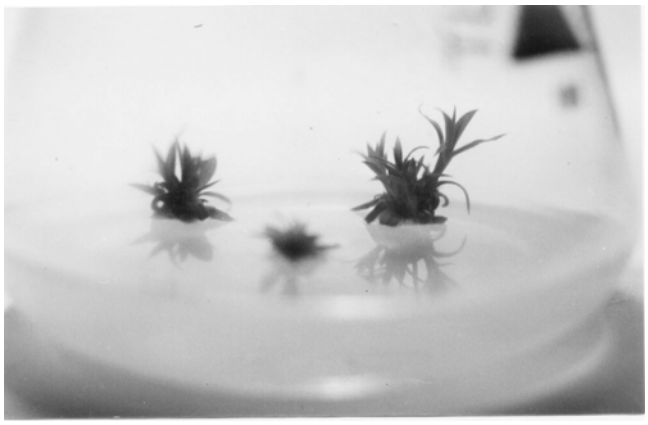

Fig. 1: Neoplantlets generated from the apices of individuals harvested in the field (Cheile Pociovaliștei); at 13 days after inoculation on V1 medium.

In the case of the second inoculation of plant material harvested in the field, the infection index, at 17 days after inoculation, was high (45%). The culture media were the same with those used for the firs inoculation. From this culture we used only the plantlets with small callus or the callus having embriogenic nods. The media on which they were transferred are the same as those used in the previous culture.

In our study we also used **seeds** harvested from Colții Vulturesei and Scărița-Belioara Reservation. The inoculation was done on a solid medium, Murashige-Skoog, diluted 50% without phytohormones. One part of the inoculated seeds were kept in light and another one in

dark. At one week after inoculation on the aseptic media, in dark, the infection index was low - 2.9%, but in light the infection index was higher - 7.6%. Both values are satisfactory, underlining the fact that good seed sterilization for *in vitro* culture can be done for this subspecies. After the germination of the seeds, the plantlets generated in dark were transferred in light. The germination rate, after 14 days from the inoculation, was 40% in dark and 33% in light. From the results mentioned above we can draw the conclusion that better results are obtained when seeds germinate in dark, but the plantlets generated in light are stronger and they are not etiolated. Apices and uni- or binodal explants obtained from seeds germination were transferred on culture media V1 and V3, without phytohormones.

The observations made during subsequent transfers, using inocula from plants harvested in the field as well as plantlets generated *in vitro* from seeds, show that the best evolution, in a prime phase, has been achieved on V1 medium, with a hormone balance of 10/1 (Fig. 2 and 3), reaching a 12.8 neoplantlets/apical inoculum. The multiplication phenomenon is also present (although at a lower scale) at a hormone balance of 1/1, i.e. V2, V3 and V4 media. After subsequent transfers on V4 medium, containing BAP (1 mg/l) and IAA (1 mg/l), a multiplication rate of 110 neoplantlets/apical inoculum was obtained.

The rhizogenesis phenomenon was present on V3 medium, at a phytohormone balance of 1/1, which is a variant where the auxins (stimulators of rhizogenesis) concentration is the highest as against to citokinins concentration. Nevertheless, the evolution of the root system was not sufficient for acclimatization. That is why the vitroplants were transferred on a ½ Murashige-Skoog culture medium, with vegetal carbon (3 g/l), without phytohormones and on other two media with phytohormones (i.e. V5 and V6). The best results were obtained on the medium with vegetal carbon, the vitroplants generating a well-developed root system, which allowed us to proceed on the next step of our study, i.e. acclimatization. For this purpose we used special caps for the culture recipients, equipped with a filter (suncap closure) having a porosity of 0.02 μm (made by Sigma). The special caps allow CO_2, from the environment, to enter the recipient. Thus, the vitroplants, which are photoheterotrophic or photomixotrophic in a normal culture, are allowed to photosyntetise, making the acclimatization process (in *ex vitro* conditions) easier [5].

Fig. 2: Plantlets of *Dianthus petraeus* ssp. *simonkaianus* generated *in vitro*, through subsequent transfers, starting from seeds harvested in Cheile Pciovaliştei; at 37 days after the transfer on V1 medium.

The vitroplants having a well-developed root system were transferred in *ex vitro* conditions using a sterilized mixture of soil-perlit-peat. Thus, their acclimatization was achieved and now they are cultivated in the area of rare, protected and endemic plants from The Botanical Garden.

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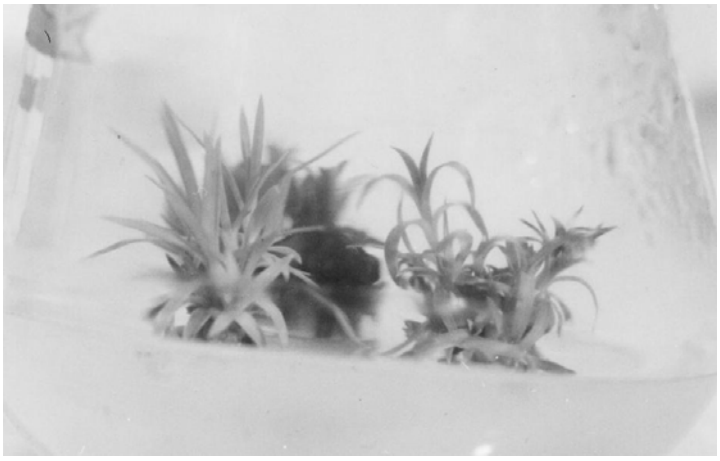

Fig. 3: Plantlets of *Dianthus petraeus* ssp. *simonkaianus* generated *in vitro*, through subsequent transfers, starting from seeds harvested in Cheile Pociovaliştei; at 37 days after the transfer on V1 medium.

A final step of our study was the inoculation of seeds harvested from each of the five locations where the subspecies is present: Scărița-Belioara Reservation, Cheile Runcului, Cheile Pociovaliştei, Valea Segăgii and Cheile Poşăgii. The seeds were selected depending on the **exposition** had by the place from where they were harvested (S,SE,SV; N,NE,NV and V). We found no individuals on eastern exposition. The aim of this last inoculation was to see if there are some differences between germination capacity of the seeds and the further development of the vitroplants, taking into consideration the exposition had by the individuals from which the seeds were harvested. All the seeds selected were fully ripened.

Table 3: Infection index and germination rate on the three different expositions

Exposition	Days after inoculation	Infection index	Germination rate
V	8	2.7%	50%
N,NE,NV			52.9%
S,SE,SV			41.41%
V	16	8.78%	81.25%
N,NE,NV			70.5%
S,SE,SV			69.6%
V	58	21.16%	87.5%
N,NE,NV			70.5%
S,SE,SV			83.83%

The infection index, at 58 days after the inoculation, allows us to consider the sterilization method as being a very good one.

Analyzing the germination rate we can see an evolution that is still present during the next phases of our study and that is the better development of plantlets originating from seeds harvested from individuals with southern and western exposition as against to northern exposition. This fact is underlined by the observations made after 58 days from the inoculation concerning the development of the plantlets. The seeds coming from individuals with northern exposition generated poorly developed and etiolated plantlets while the plantlets generated from the seeds of the individuals with southern and western exposition were vigorous, having up to 6 internods (Fig. 4 and 5).

Fig. 4: Plantlets of *Dianthus petraeus* ssp. *simonkainaus* generated from seeds sterilized and inoculated *in vitro* (southern exposition).

Fig. 5: Plantlets of *Dianthus petraeus* ssp. *simonkainaus* generated from seeds sterilized and inoculated *in vitro* (western exposition - small flasks; northern exposition – the bigger flask).

The plantlets were cut and the minicuttings resulted (Fig. 6) were transferred on two culture media (V10 and V11) with the purpose of obtaining a multiplication rate as high as possible and to induce rhizogenesis. The exposition of the plants from which the seeds were harvested had been taken into consideration again. The observation made at 32 days after the transfer are presented in table 4.

From the above table it can be seen that the V11 medium favors multiplication while the V10 medium stimulates the elongation of the plantlets. The differences between plantlets concerning the exposition are still present, the vitroplants coming from northern exposition being smaller and having the lowest multiplication rates.

The existent differences between seeds harvested from individuals living on places with different insolations, in terms of germinating capacity, can be explained however by the different degree of maturation, although apparently ripened seeds were inoculated.

Fig. 6: Apical and nodal explants, generated *in vitro* from plants of *Dianthus petraeus* ssp. *simonkaianus*; at 12 days after inoculation.

Table 4: Multiplication rate and the average length of the vitroplants at 32 day after the transfer.

Exposition	Culture medium	Multiplication rate	The average length of the vitroplants (mm)
S,SE,SV	V11	2.25	19.55
	V10	1.8	22.2
V	V11	2.16	17.6
	V10	1.3	32.4
N,NE,NV	V11	1.58	19.4
	V10	1.15	21

Conclusions

The experiments carried out for this subspecies have led to its micropropagation without special difficulties. Through subsequent transfers a multiplication rate of 110 neoplantlets/inoculum was achieved. The best culture medium has proven to be the one with a hormone balance of 10/1 (1mg/l BA/0.1 mg/l ANA). The rhizogenesis phenomenon appears on the medium with 1 mg/l NAA and 1 mg/l K but the medium containing vegetal carbon has proven to be the best. A satisfactory aclimatization of the vitroplants was done. Taking into consideration the role played by the exposition from which the explant originates (in this case, seeds) the best results are obtained for southern and western exposition.

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MICROPROPAGAREA LA *DIANTHUS PETRAEUS* W. ET K. SSP. *SIMONKAIANUS* (PÉTERFI) TUTIN

(Rezumat)

Biotehnologia culturilor vegetale *in vitro* reprezintă o modalitate eficientă de multiplicare a speciilor de plante rare, vulnerabile sau periclitate. *Dianthus petraeus* W. et K. ssp. si*monkaianus* (Péterfi) Tutin se întâlneşte pe staţiuni calcaroase de pe teritoriul ţării noastre (Masivul Gilău-Muntele Mare) şi în ţări precum Macedonia, Bulgaria sau Grecia [1,3,8]. Populaţiile din ţara noastră sunt din ce în ce mai mult afectate de exploatările de calcar, păşunatul excesiv şi recoltarea plantei ca ornament. Astfel, această subspecie apare menţionată în Listele Roşii elaborate pentru flora României ca fiind rară [6,7,8]. În vederea micropropagării, aceastei subspecii de *Dianthus petraeus* a fost folosit material vegetal recoltat din Masivul Gilău-Muntele Mare în anii 2001 şi 2002. Materialul vegetal folosit la inducerea culturilor aseptice a constat din apexuri, explante apicale şi nodale (uni- sau plurinodale), noduri florifere, muguri şi seminţe. Inducerea culturilor s-a realizat cel mai uşor folosind ca material iniţial explante apicale şi nodale (uni- sau plurinodale), în timp ce mugurii florali nu au regenerat. Culturile generate din seminţe au urmărit, în principal, influenţa pe care o are expoziţia la soare a staţiunilor pe care se dezvoltă indivizii de la care s-a recoltat materialul vegetal, precum şi influenţa pe care o are lumina/întunericul asupra ratei de germinaţie, respectiv de infecţie. Rezultatele au aratat că expoziţia nordică este evident defavorizată în comparaţie cu expoziţiile sudică şi vestică. Mediul de cultură folosit a fost un mediu bazal cu mai multe variante, în funcţie de fitohormonii adăugaţi. Fitohormonii utilizaţi au fost: ANA (acid naftilacetic), AIB (acid indolilbutiric) şi AIA (acid indolilacetic), ca auxine, iar ca citochinine – BAP (6 – benzilaminopurina), kinetina (6 – furfurilaminopurina), 2iP (6 - dimetilaminopurina) şi TDZ (tidiazuron). Modificându-se balanţa fitohormonală (raport citochinine/auxine) s-a urmărit obţinerea unei rate crescute de multiplicare în paralel cu inducerea rizogenezei. Pe mediul de cultură conţinând benziladenină (1 mg/l) şi acid indolilacetic (1 mg/l) s-a obţinut o rată de multiplicare de până la 110 neoplantule/inocul apical. Rizogeneza a fost puternic stimulată pe un mediu de bază Murashige-Skoog diluat 50%, conţinând cărbune vegetal 3g/l, rezultate mulţumitoare obţinându-se şi pe mediile cu 2iP şi TDZ. Vitroplantele obţinute, cu sistem radicular bine conformat, au fost trecute în condiţii *ex vitro,* într-un amestec sterilizat de pământ-perlit-turbă, realizându-se aclimatizarea acestora. Pentru realizarea unei bune aclimatizări folia de polietilenă, ce izolează aproape ermetic vasul de cultură faţă de mediul ambiant, a fost înlocuită cu o folie de polietilenă prevăzută cu un disc filtrant care permite trecerea CO_2. Astfel, vitroplantele au trecut de la un regim heterotrof la unul autotrof, şocul cauzat de condiţiile *ex vitro* fiind mult atenuat [5].

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