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INFLUENCE OF HORMONE BALANCE AND *IN VITRO* PHOTOAUTOTROPHY ON *DIANTHUS SPICULIFOLIUS* SCHUR MICROPROPAGATION

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Abstract: The biotechnology of *in vitro* cultures 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 spiculifolius* Schur is a Carpathian endemism. Due to excessive anthropization in some regions of its distribution area, the populations in question are declining. At present, the species is mentioned in the various Red Lists elaborated for rare and vulnerable Romanian flora species [6,9]. The aseptic culture was induced starting from plant material harvested from the Piatra Craiului Massif. The inocula consisting of apical and nodal explants were cultured on aseptic media, with a varied hormone balance, in order to obtain an as high as possible multiplication rate and induction of rhizogenesis. The multiplication rate was increased from 7.5 new plantlets/inoculum to 31.8 new plantlets/inoculum, and an adequate rhizogenesis rate was obtained at the same time. Micropropagation and acclimatization were favored by the *in vitro* photoautotrophic culture.

Introduction

The biotechnology of *in vitro* cultures is increasingly used as part of programs for the *ex situ* and *in situ* conservation of numerous species of the spontaneous flora that are endemic, endangered, vulnerable or rare. *Dianthus spiculifolius* Schur is a Carpathian endemite, found in both the Romanian Carpathians (Apuseni Mountains, Meridional Carpathians and Oriental Carpathians) as well as in the Ukrainian Carpathians [4,10,11]. It grows on the calcareous rocks of the mountain belt up to the subalpine belt. It is a perennial, caespitose plant, a "pad" bearing several floral stems up to 30 cm high. Flowers appear in June-August, they are white or pale rose. Due to excessive anthropization in some regions of its distribution area (lime quarries, pasturing, tourism, harvest due to its special ornamental potential etc.) the populations in question are declining. At present, the species is mentioned in the various Red Lists elaborated for rare and vulnerable Romanian flora species [6,9].

This species was studied by other authors [1,2,3,12,13], who multiplied it *in vitro* and studied its genetic variability, before and after its introduction *in vitro*.

Although some authors [1,12] have reported a culture medium on which new plantlets are completely organized, i.e. the medium containing 2-3 g/l vegetal coal, multiplication on this medium has not been the highest one. The authors mentioned above have also investigated different culture media, the best results being generally obtained by using cytokinin 2 iP (2-isopentenyladenine). This paper aims to achieve a protocol of aseptic culture and to set an optimum phytohormone balance (cytokinins/auxins) that may allow the micropropagation and acclimatization of this rare species of our flora, at a low cost and with a satisfactory multiplication rate, with a future view to creating plant material used for its *in situ* repopulation and also for its *ex situ* culture.

Material and methods

The aseptic culture was induced starting from plant material harvested from the Piatra Craiului Massif in the summer of 2000. The plants were vegetating on grassy shelves, near to Marele Grohotiş, at an altitude of approximately 1800 m. Inocula consisting of apical and uninodal explants were cultured on aseptic media, with a varied hormone balance. The culture media contained macroelements, microelements and FeEDTA according to Murashige-Skoog (1962), supplemented with vitamins (thiamine HCl, pyridoxine HCl and nicotinic acid, 1 mg/l each), myo-inositol (100 mg/l), saccharose (20 mg/l) and agar 7 g/l. The hormone balance was set so as to obtain an as high as possible multiplication rate and induction of rhizogenesis (Tab. 1). The culture was initiated on medium V1. Subsequently, the explants were transferred to media V1, V2, V3 and V4.

Table 1: Variants of culture media used for the induction of aseptic cultures and micropropagation in *Dianthus spiculifolius* Schur.

Variant	Cytochinin/auxin ratio	Phytohormone concentration (mg/l)
V1	1/1	K= 1 ANA= 1
V3	2.5/1	BA= 2.5 ANA= 1
V4	5/1	BA= 5 ANA= 1
V2	10/1	BA= 1 ANA= 0.1

The phytohormones used were ANA (naphthaleneacetic acid) as an auxin, and kinetin (6-furfurylaminopurine) and BA (6-benzylaminopurine) as cytokinins. In the composition of culture media, we considered the use of phytohormones with the lowest cost. Thus, the cost of kinetin, initially used for the induction of the

aseptic culture, is 2.5 times higher than the cost of BA that we considered to subsequently use instead of kinetin. The cost of 2iP with which other authors have obtained very good results [1] is 5.4 times higher than the cost of BA.

The sterilization of the plant material harvested in the field was done with Domestos 100%, for 15 minutes. The cultures were maintained at a temperature of 25±2ºC, at a light intensity of 87 μmol/m^2/s and a photoperiod of 16 h light/8 hours dark.

Results and discussion

At 43 days after the inoculation of plant explants consisting of apices and uni- or binodal fragments (approx. 1 cm) on medium V1, the infection index was 50% and the regeneration rate 30%. In the case of this species, this initial rate of induction of an aseptic culture can be considered satisfactory, as subsequently, through uninodal and apical explants, good multiplication was obtained. Thus, in the period 2 August – 19 December (132 days), from two apices introduced *in vitro*, approximately 150 vitroplants were obtained by minicuttings, after only two transplantations.

Subsequently, apical and binodal explants of the new plantlets were transferred to media V1 (phytohormone balance 1/1) and V2 (phytohormone balance 10/1). Medium V2 has a cytokinin concentration 10-fold higher that that of auxins compared to the first medium, in order to stimulate multiplication (Tab. 2 and Fig. 1).

Table 2: Influence of the content of phytohormones and their ratio on *Dianthus spiculifolius* Schur minicuttings; observations performed 28 days after inoculation.

Variant	cytokinin/auxin ratio	Phytohormone concentration (mg/l)	Observations
V1	1/1	K= 1 ANA= 1	**- multiplication rate = 7.5 new plantlets/inoculum** - shorter internodia - longer leaves (5 cm) - more chlorophyll - good rhizogenesis - weak callusogenesis
V2	10/1	BA= 1 ANA= 0.1	- **multiplication rate = 9.3 new plantlets/inoculum** - lower growth - rhizogenesis present - callusogenesis

As it is shown in Figure 1, apical explants have a better evolution compared to nodal ones. However, nodal explants also have a good evolution and show the

multiplication phenomenon. During this first stage, a multiplication rate of 7-9 new plantlets/inoculum was obtained.

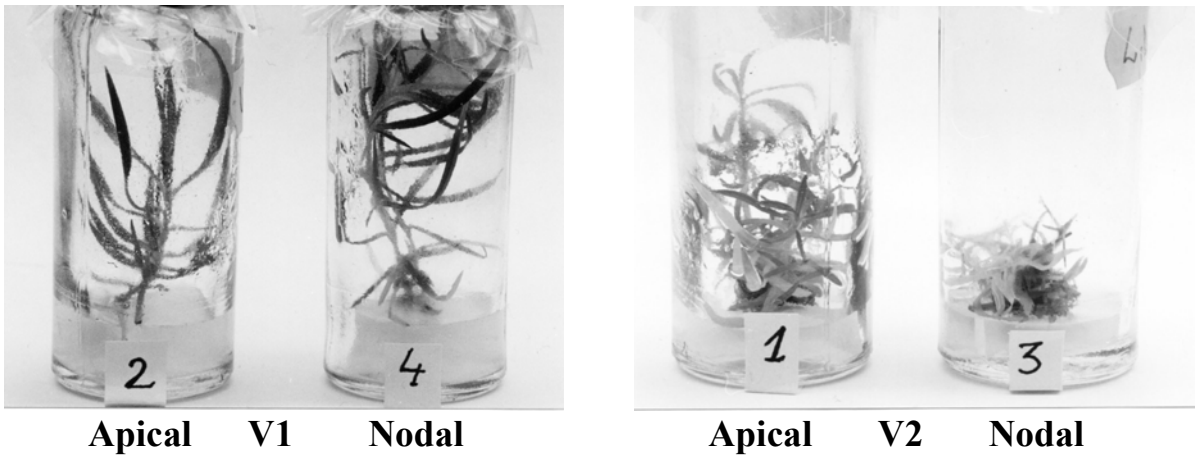

Fig. 1. The evolution of apical and nodal explants of *Dianthus spiculifolius* Schur, on V1 and V2 media; 56 days after inoculation.

On medium V1, where the cytokinin/auxin ratio is 1/1, the generated plantlets are vigorous, rhizogenesis is well represented, and the assimilating pigments content of the leaves is higher (Tab. 3). The leaves of the plantlets grown on a culture medium containing kinetin as well as cytokinin, has a 3.5-fold higher chlorophyll content (*a*+*b*) and a 3.8-fold higher carotenoid pigment content, compared to the leaves of plantlets grown on the BA medium. In fact, the kinetin phytohormone is well known to increase the chlorophyll level, the number of chloroplasts/cell and the degree of development of thylakoids, in tissues cultured *in vitro* [7]. In the presence of a 1/1 hormone balance, when auxins have a relatively high concentration compared to that of cytokinins, rhizogenesis is well represented.

Table 3: Influence of cytokinins (kinetin and BA) on the assimilating pigment content (µg/g SP) in the *Dianthus spiculifolius* Schur vitroplant leaves.

Variant	Phytohormone concentration (mg/l)	chl. *(a+b)* (µg/g FW)	chl. *a/b*	carot. (µg/g FW)	total assimil. pigm. (µg/g FW)
V1	K= 1 ANA= 1	922	1.52	159	1081
V2	BA= 1 ANA= 0.1	3258	1.37	606	3864

In the case of medium V2, where the cytokinin/auxin ratio was increased to 10/1, in order to obtain a better multiplication, a slight increase in the multiplication rate was obtained and rhizogenesis was present.

Then, the influence of a hormone balance (cytokinins/auxins) of 2.5/1 (medium V3) and 5/1 (medium V4) was investigated (Tab. 4 and Fig. 2).

Table 4: Influence of hormone balance on micropropagation in *Dianthus spiculifolius* Schur; observations performed 45 days after inoculation.

Variant	cytokinin/auxin ratio	Phytohormone concentration (mg/l)	Observations
V1	1/1	K= 1 ANA= 1	- good further development - multiplication rate = 16.4 new plantlets/inoculum - the best rhizogenesis - weak callusogenesis
V3	2.5/1	BA= 2.5 ANA= 1	- good development - multiplication rate = 12 new plantlets/inoculum - no rhizogenesis - medium callusogenesis - tendency for vitrification in some inocula
V4	5/1	BA= 5 ANA= 1	- medium development - multiplication rate = 11.3 new plantlets/inoculum - no rhizogenesis - medium callusogenesis - tendency for vitrification in some inocula
V2	10/1	BA= 1 ANA= 0.1	- the best development - multiplication rate = 31.8 new plantlets/inoculum - medium rhizogenesis - medium callusogenesis

Following this transfer, in the case of a 1/1 phytohormone balance, a multiplication rate of 16.4 new plantlets/inoculum was obtained, and for a balance of 2.5/1 and 5/1 the multiplication rate was 11.3-12 new plantlets/inoculum. The best development was noted in the case of a phytohormone balance of 10/1, when a multiplication rate of 31.8 plantlets/inoculum was obtained. The fact that the best rhizogenesis results at 1/1 cytokinin/auxin ratio is due to the increased auxin amount, compared to cytokinin. Auxins are known to favor both *in vitro* and *ex vitro* rhizogenesis.

Since the highest multiplication rate is obtained on medium V2 that contains the lowest phytohormone amount, due to an optimum hormone balance, we could recommend the use of this type of culture medium for the efficient micropropagation of this species.

Fig. 2: The evolution of *Dianthus spiculifolius* Schur minicuttings on diferrent culture media; 44 days after inoculation:

With a view to an optimum acclimatization of the vitroplants obtained, *in vitro* photoautotrophic cultures were used (Fig. 3).

Fig. 3: Photoautotrophic *Dianthus spiculifolius* Schur culture; 12 days after inoculation (arrow indicates the suncap closure).

Although photoautotrophy is characteristic of the vast majority of cormophytes, due to specific conditions, these plants are heterotrophic or photomixotrophic *in vitro.* The tight closure of the culture recipients, in order to prevent the infection of cultures, leads to the consumption of CO_2 from the atmosphere of the recipients during the first hours after their closure. This is why in order for the inocula to grow, they need an organic carbon source (usually saccharose). *In vitro* photoautotrophic cultures are generally performed on saccharose free culture media and the atmosphere of the culture recipients is supplemented with approx. 2% sterilized CO_2 using special caps equipped with a filter (suncap closure) [5]. Thus, the inocula can develop even *in vitro* on

photosynthetic bases, being less affected at the time of the *ex vitro* acclimatization (Tab. 5, Fig. 3).

Table 5: Evolution of photoautotrophic vitroplants compared to those classically cultured in vitro.

Variant		Length of new plantlet (mm)	Multiplication rate
Saccharose concentration	CO_2		
Classic		41	8.3
2 %	0 %		
Photoautotrophic		64	12.2
0 %	***2 %***		

Dianthus spiculifolius new plantlets cultured under photoautotrophic conditions (on saccharose free culture media, with the atmosphere of the recipients supplemented with 2% CO_2) have a 56.1% higher length and a 47% higher multiplication rate than those classically cultured under aseptic conditions (on media with 2% saccharose and no CO_2 supplementation).

The acclimatization of vitroplants was carried out in a proportion of 80% for photoautotrophic vitroplants and 60% for those classically cultured *in vitro.*

Conclusion

The studies performed resulted in an increase in the *in vitro* multiplication rate of this species, from 7.5 new plantlets/inoculum to 31.8 new plantlets/inoculum, and a concomitant rhizogenesis corresponding to acclimatization. The most favorable culture medium for micropropagation and at the same time the most cost-efficient one was the medium containing BA 1 mg/l and ANA 0.1 mg/l, i.e. with a phytohormone balance of 10/1. The photoautotrophic culture of vitroplants preceding their transfer from *in vitro* to *ex vitro* conditions, favors the acclimatization process.

The studies for the micropropagation of this rare species of our flora, with special ornamental potential, were performed in order to introduce the plant in culture systems, as another form of *ex situ* conservation.

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INFLUENŢA BALANŢEI HORMONALE ŞI A FOTOAUTOTROFIEI ASUPRA MICROPROPAGĂRII LA *DIANTHUS SPICULIFOLIUS* SCHUR

(Rezumat)

Biotehnologia culturilor *in vitro* este tot mai mult utilizată în cadrul programelor de conservare *ex situ* şi *in situ* a multor specii de plante cu diverse grade de periclitare sau endemice din flora spontană.

Dianthus spiculifolius Schur este un endemit carpatic întâlnindu-se atât în Carpaţii româneşti (M-ţii. Apuseni, Carpaţii Meridionali şi Carpaţii Orientali), cât şi în Carpaţii ucrainieni. Creşte pe stâncării calcaroase din etajul montan până în cel subalpin. Datorită antropizării excesive din unele regiuni ale arealului său (exploatări de calcar, păşunat, turism, recoltarea plantei datorită valenţelor ornamentale deosebite etc.) populaţiile respective se găsesc

în declin. În prezent, specia apare menţionată in diversele Liste Roşii elaborate pentru flora României ca rară [9] şi vulnerabilă [6].

Inducerea culturii aseptice s-a realizat, pornind de la material vegetal recoltat din Masivul Piatra Craiului, în vara anului 2000. Inoculii constând din explante apicale şi nodale au fost cultivaţi pe medii aseptice, cu o balanţă hormonală variată, urmărindu-se obţinerea unei rate de multiplicare cât mai mare şi inducerea rizogenezei. Pe mediul de cultură conţinând 1mg/l BA şi 0,1mg/l ANA, respectiv o balanţă hormonală citokinină/auxină 10/1 s-a reuşit mărirea ratei de multiplicare de la 7,5 neoplantule/inocul, la 31,8 de neoplantule/inocul, obţinându-se concomitent şi o rată corespunzătoare de rizogeneză. Micropropagarea şi aclimatizarea au fost favorizate de culturile *in vitro* fotoautotrofe.

Studiile efectuate în vederea micropropagării acestei specii rare pentru flora noastră dar în acelaşi timp şi cu valenţe ornamentale s-au realizat cu scopul introducerii plantei în cultură, ca o altă formă de conservare *ex situ*.