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DIANTHUS GIGANTEUS SUBSP. *BANATICUS*: ECO-
COENOTIC AMBIANCE AND *EX SITU*
CONSERVATION METHODS

Abstract of PhD thesis

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Key words: *Dianthus giganteus* subsp. *banaticus*, endemic, eco-coenotic ambiance, *ex situ*, *in vitro*, cryopreservation, SSR, ISSR molecular markers, somaclonal variability.

Introduction

The efficient approach of plants conservation implies the use of *in situ* and *ex situ* methods, having as a main target the maintenance of an appropriate genetic diversity of the concerned species. *Ex situ* conservation is a viable alternative in case one taxon is highly endangered in its natural environment, or may complete the *in situ* conservation methods.

The aim of this PhD thesis is to study *Dianthus giganteus* subsp. *banaticus* taxon in its natural environment and to *ex situ* conserved it.

The main **objectives** of the thesis are:

- description of eco-coenotic ambiance of *Dianthus giganteus* ssp. *banaticus* taxon;
- *in vitro* cultivation of the taxon and establishing a micropropagation protocol with optimum efficiency;
- cryopreservation (in liquid nitrogen, at -196°C) of the micropropagated material for long term storage;
- checking the appearance of a possible somaclonal variability induced by *ex situ* conservation methods.

The proposed subject is part of a more extensive research project, PN II 31-008/2007 (acronym CONEXITVARPER, manager CS II Dr. Victoria Cristea), entitled **“Consolidation of biodiversity by *ex situ* conservation and assessment of somaclonal variability by means of genomic analysis molecular techniques at endemic/endangered taxa in NATURA 2000 sites”**. This project was conducted in

Research program 4: “Partnerships in priority fields”, subfield “Scientific substantiation, projection and development of Natura 2000 protected areas network in Romania, as well as the adaptive management plans, which guarantee the conservation of biologic and ecologic diversity”. The financial support during doctoral research was offered by BBU scholarship within POSDRU 6/1.5/S/3 project – “DOCTORAL STUDIES: THROUGH SCIENCE TOWARDS SOCIETY”.

1.1 Description and chorology of *Dianthus giganteus* ssp. *banaticus* taxon

The studied taxon, *Dianthus giganteus* D’Urv. subsp. *banaticus* (Heuff.) Tutin, belongs to *Caryophyllaceae* family, and is endemic for the South-West Carpathians, considered vulnerable in Romania by some botanists (Dihoru and Dihoru, 1994, Sârbu et al., 2003), and rare by others (Olteanu et al., 1994). It was described for the first time in „*Enumeratio Plantarum in Banatu Temesiensi sponte crescentium et frequentius cultarum*” (1858), by Heuffel as follows: capitulum with numerous dense flowers, involucre scales with curled edges, calyx of 1.5 cm length, petals lamina blood-colored or purple, four angular stem, rough on the inferior part. Heuffel did not fit the taxon within *D. giganteus*, but *D. carthusianorum*.



Fig.1. *D. g.* subsp. *banaticus*, 2009 June, in Mraconiei Valley (photo. L. Jarda).

Dianthus giganteus subsp. *banaticus*, or Banat carnation, is a perennial plant of 25-60 cm height, with petals lamina blood-colored or purple, which blooms in June-July. It grows in meadows, on screes and rocks, and skeletal limestone soil in Caraş-Severin and Mehedinţi counties. Sometimes, this species resembles very well with *Dianthus giganteus* subsp. *giganteus* that it may be mistaken with, especially when the calyx size is the same and the color is reddish not green, as usual. In this case, the difference

between the two is done by the internal calyx scales, as follows: in case of *banaticus* the scales are aristated, while in *giganteus* are more acute or acuminate. Also, the fewer flowers, petals two times bigger and with hairs at *banaticus* are other differences between the two species, or subspecies, respectively (Prodan, 1953).

The status of this taxon is species, according to Ciocârlan (2009): *D. banaticus* (Heuffel) Borbás, but we kept the one from **Flora Europaea**. Although, according to Oprea (2005) the taxon is cited from Caraș-Severin, Cluj, Brașov, Iași, Mehedinți, and Sibiu counties, its presence is confirmed only in Caraș-Severin and Mehedinți counties (Dihoru and Pârvu, 1987; Ciocârlan 2009; Sârbu et al., 2003). It is likely that this subspecies was mistaken with other *Dianthus* species, and the data were cited as such in the future publications (Dihoru and Pârvu, 1987; Oprea, 2005). Regarding the ambiance of *Dianthus giganteus* subsp. *banaticus* it was included, according to diverse bibliographic sources, into 4 plant communities: *Cystio-Festucetum rupicolae* Peia 1981, *Stachyo nitens-Cachrysetum ferulaceae* Sanda et Popescu, 1999 (Matacă, 2005); *Melico-Phleetum montani* Boșcaiu et al., 1996; *Acantho longifolii-Quercetum pubescentis* Jakucs et Fekete, 1958 (Matacă, 2003).

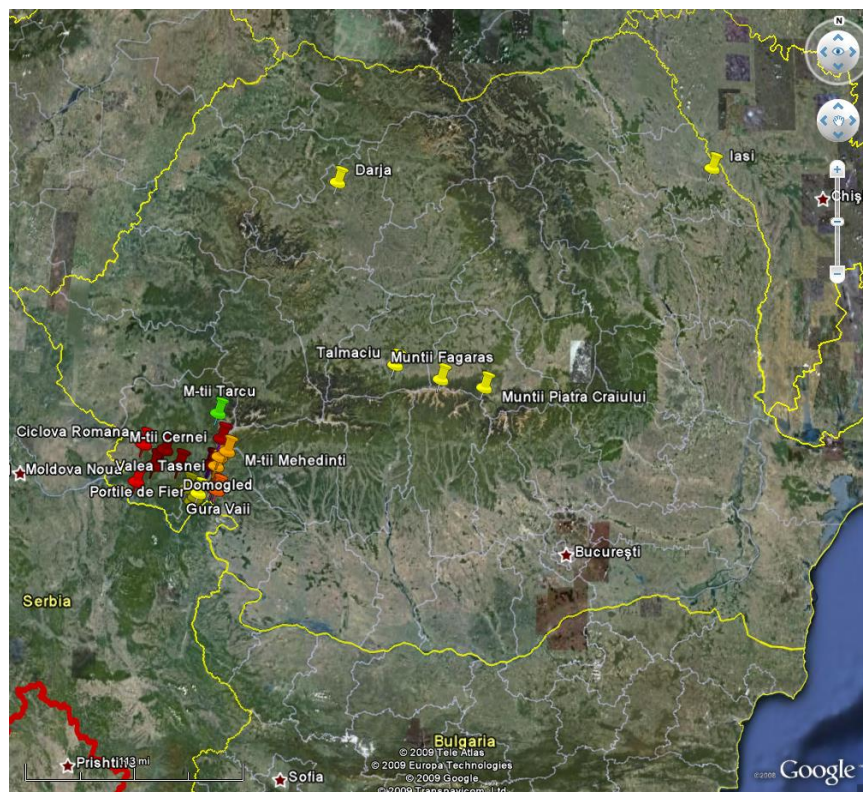


Fig. 2. Distribution of *D. g.* subsp. *banaticus* taxa, (according to A.Oprea, 2005).

The development of society and the growing intensity of human activities impact on nature, cause the destabilization of regional and global ecologic balance. Plants play a

very important role in maintaining this balance, but their uncontrolled exploitation may cause, besides losing the ecological balance, the damage of plant genofond. Knowledge about some ambient factors, as well as the relationships between different plant communities makes possible a rational exploitation of plant resources.

For this reason, we took into consideration the eco-coenotic ambience of the studied taxa, insisting on estimation of the growth and development degree, as an indication of the relationships between its ecological requirements and the diverse habitats where it can be found.

2. MATERIAL AND METHODS

2.1. Eco-coenotic ambience of *Dianthus giganteus* subsp. *banaticus* taxon

- phytosociologic relevés were done according to Braun-Blanquet method, at least 2 relevés/population (subpopulation);
- the relevés technique and the quality and quantity assessment were done according to Borza and Boşcaiu (1965) and Cristea, Gafta and Pedrotti (2004);
- the analyzed surface was 25m² for each relevé;
- the 24 personal phytosociologic relevés were analyzed and included in plant communities according to Cluj school methodology.

2.2 *Ex situ* conservation of *Dianthus giganteus* ssp. *banaticus* taxon

- was done outdoor and on a special rock in “Al. Borza” Botanical Garden, as well as *in vitro* culture and cryopreservation.

a. *In vitro* classical culture

For the **initiation** of *in vitro* culture of *Dianthus giganteus* subsp. *banaticus* taxa, plant material was sterilized using several methods, as described in Table 1. Before sterilization, the plant material was pre-sterilized by washing in water, rinsing in Domestos 5%, followed by a short rinse in ethylic alcohol 80%.

Table 1. Sterilizing methods used for plant material disinfection

Sterilizing method	Shoots/flowering stems
a	10 min. Domestos 20 %
b	10 min. HgCl ₂ 0,2 %
c	5 min. Domestos 10 %, 5 min. HgCl ₂ 0,2 %

After 30 days from initiation, the explants were transferred on multiplication-stabilization medium (D2), which contains Murashige&Skoog basal medium with vitamins, with 20 g/l sucrose and a hormonal balance favoring cytokinines (BAP 1mg/l, NAA 0,1 mg/l), for stimulating the multiplication.

The plants obtained by *in vitro* culture were used for optimum multiplication rate and rhizogenesis studies. We used different culture media, with hormonal balance favoring cytokinines for **multiplication** and favoring auxines for **rhizogenesis**, and Murashige&Skoog medium with vitamins for the control (Tabs. 2 and 3). The cultures growth was at 24°C, in photoperiodic schedule of 16h of light at an intensity of 3000 – 3500 lux.

Table 2. Variants of culture media used for optimum multiplication

Components		Variants								
		M1	M2	M3	M4	M5	M6	M7	M8	M9
Macroelements, microelements, and vitamins		MS	MS	MS	MS	MS	MS	MS	MS	MS
Hormones (mg/l)	NAA	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	-
	K	0,1	1	-	-	-	-	-	-	-
	BAP	-	-	0,1	1	-	-	-	-	-
	2iP	-	-	-	-	10	15	-	-	-
	TDZ	-	-	-	-	-	-	0,01	0,05	-
Sucrose (g/l)		20	20	20	20	20	20	20	20	20
Agar (g/l)		7,8	7,8	7,8	7,8	7,8	7,8	7,8	7,8	7,8

Table 3. Variants of culture media used for optimum rhizogenesis

Components		Variants			
		R1	R2	R3	R4
Macroelements, microelements, and vitamins		MS	MS	MS	MS
Hormones (mg/l)	IAA	-	-	-	1
	NAA	-	0,1	1	-
	K	-	0,5	0,5	0,5
Sucrose (g/l)		20	20	20	20
Agar (g/l)		7,8	7,8	7,8	7,8

b. *In vitro* photoautotrophic culture and microscopy

For the **photoautotrophic (PA)** cultures explants from *in vitro* classical culture were used, obtained after 12 months from culture initiation. The medium was Murashige&Skoog basal medium with vitamins and hormones (NAA – 0.1 mg/l; K – 0.5 mg/l), but without sucrose for the PA variant and with 20 g/l sucrose for the control. Erlenmeyer 100 ml bottles were used. The bottles for the PA culture were covered with “suncap” (polypropylene foil with 6 mm diameter filter and 0.02 µm porosity), which allows the gases exchange with the environment, but does not allow the entrance of pathogenic agents. For the control, the bottles were covered with regular polypropylene foil. There were inoculated 4 explants/culture vessel. The system was maintained in the vegetation room at a temperature of 25±1°C, a photoperiod of 16 h of light/8 h of dark and an intensity of 4500-5000 lux.

The material used for **microscopy** was represented by:

- i) control group – leaflets coming from one individual from the Botanical Garden;
- ii) *in vitro* culture group – leaflets coming from the same individual obtained by *in vitro* classical culture;
- iii) PA culture group – leaflets coming from the same individual obtained in photoautotrophic culture (0.03% CO₂).

There were done structural observations (light microscopy) for the entire mesophyll of the leaf, as well as ultrastructural investigations (electronic microscopy) for detailed observations, regarding mainly the chloroplasts.

c. Acclimatization

The plant material obtained by *in vitro* culture, after the photoautotrophic pre-acclimatization phase, was transferred to *ex vitro* culture, acclimatized to the conditions outside the vessels: reduced humidity than *in vitro*, other culture substratum, other composition of the atmosphere, the major influence of ambient factors, etc. These were transferred on two substrata variants: perlite and perlite+soil (50%-50%). The next phase in the acclimatization process was the transfer of plants from the two substrata variants to a single substratum, soil, in larger pots maintained in heated greenhouse. After a period of 30 days from the culture in protected environment (in greenhouse), we considered plants were stable and able to be transferred outdoor. The plants were taken into hotbeds and were maintained in pots for another 30 days, after that being transferred on a rock in “Al. Borza” Botanical Garden.

d. Cryopreservation

Apical meristems with 2-4 foliar primordia, having 3-4 mm length, were used for cryopreservation experiments (=stem apexes). The sore cultures of *in vitro* plants were grown in 500 ml polypropylene containers on modified Murashige and Skoog (1962) (MS) medium. The nutritive medium composition was: macro- and microelements MS; 0.1 mg/l BAP; 0.01 mg/l NAA; 30 g/l sucrose and 7 g/l agar. The pH was adjusted to 5.7 before autoclaving. The cultures growth was done at 24°C in a photoperiodic schedule of 16 h of light at an intensity of 3500 lux.

The same medium, with less agar (5 g/l agar), was used for the regeneration of plants after cryopreservation.

The apexes were isolated in sterile conditions at a stereomicroscope with sterile needles. They were incubated in Petri plates (5 mm in diameter) on filter paper wetted with 2.5 ml liquid medium, for 24 h, at 24°C. The apexes were then incubated for 24 h in MS media with sucrose in different concentrations (0.25, 0.5, and 0.75 M). The incubation was done in Petri plates (5 mm in diameter) on filter paper wetted with the above mentioned sucrose solutions.

The apexes were then treated with PVS2 vitrification solution (Sakai et al., 1990) for 10-30 minutes at room temperature. The apexes were transferred on aluminium foil (2 x 0.5 cm length, previously sterilized), and then were transferred in 2 ml polypropylene tubes to be frozen.

The freezing was done by direct immersion in liquid nitrogen of the tubes containing the apexes. After 2 h of storage in liquid nitrogen, the probes were liquefied in liquid medium at room temperature.

After liquefying, the apexes were transferred on semisolid culture media (5 g/l agar). The storage for regeneration purposes was done in the above mentioned light and temperature conditions, for growing and multiplication of *in vitro* plants.

2.3 Study of somaclonal variability in *Dianthus giganteus* subsp. *banaticus*

The material used in molecular studies was represented by leaflets of individuals (i) before the initiation of *in vitro* cultures, (ii) after maintenance for 24 months in culture on different culture media (Tabs. 2 and 3). Genomic DNA isolation from leaves was done using CTAB according to Doyle and Doyle (1987). Two molecular marker types ISSR (Inter Simple Sequence Repeat) and SSR (Simple Sequence Repeat) were used.

3. RESULTS AND DISCUSSIONS

3.1 Eco-coenotic ambiance of *Dianthus giganteus* ssp. *banaticus* taxon

There were identified 10 plant communities where the studied taxon can be found, which belong to 5 vegetation classes that emphasize the wider ecologic amplitude of this taxon, against the only 4 plant communities mentioned in the literature:

- A. *Agrostio tenuis-Festucetum rupicolae* Csürös-Káptalan (1962) 1964
- B. *Agrostio-Danthonietum provincialis* Soó 1947
- C. *Arrhenatheretum elatioris* (Br.-Bl. 1919) Scherrer 1925
- D. *Thymo pannonici-Chrysopogonetum grylli* Doniță *et al.*, 1992
- E. *Festucetum rupicolae* Burduja *et al.*, 1956, Klika, 1931
- F. *Rhinantho rumelici-Brometum erecti* Sanda *et* Popescu, 1999
- G. *Achnatheretum calamagrostis* Br.-Bl. 1918
- H. *Thymo comosi-Galietum albi* Sanda *et* Popescu 1999
- I. *Festucetum xanthinae* Boșcaiu 1971
- J. *Cotino-Carpinetum orientalis* Csürös *et al.* 1968

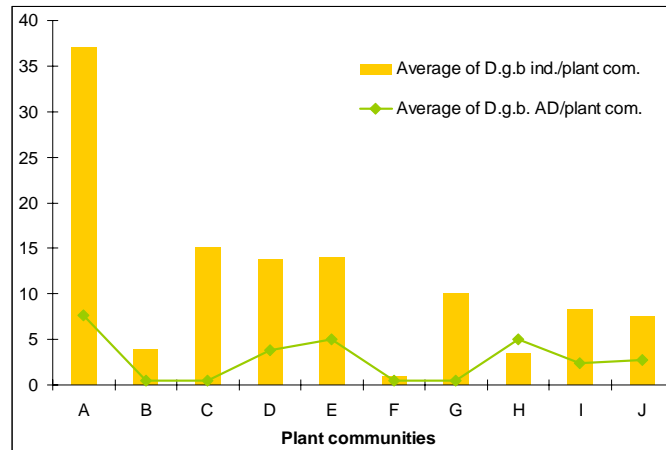


Fig. 3. The average number of *Dianthus g. subsp. banaticus* individuals and AD average in the studied plant communities

The taxon is most frequently encountered in *Agrostio tenuis-Festucetum rupicole* (A) plant community, and uncommon in *Rhinantho rumelici-Brometum erecti* (F) (Fig. 3). In *Thymo comosi-Galietum albi* (H) plant community it can be observed that the abundance-dominance is greater than the number of individuals in phytocoenoses. This is due to the fact that individuals have a good growth and development with many flowering stems, having a wider covering.

3.2 *Ex situ* conservation of *Dianthus giganteus ssp. banaticus* taxon

a. *Ex situ* conservation in the phytogeographic department of “Al. Borza” Botanical Garden

A rocky area was used for establishing *ex situ* collection in the Botanical Garden. When possible, the pick up of individuals from the wild was done with the afferent soil and then was planted in the Botanical Garden. There were planted individuals of *D. giganteus* subsp. *banaticus* from 3 populations, 3 individuals from each population, 9 individuals on the whole, in case of this taxon. Some individuals that were picked up with flowers or buds formed seeds. Others bloomed on the rock and had viable seeds; these were taken and put in the germplasm database of the botanical garden. Among the 9 planted individuals, there are still 8 on the special rock, which bloom and have seeds every year since 2008 (Fig. 4).



Fig. 4. *D. g.* subsp. *banaticus* after one year (2009) from the plantation in the Botanical Garden

b. Classical *in vitro* culture

Ex situ multiplication and reinsertion into the natural habitats may contribute to the maintenance, consolidation and even extension of the natural populations. On the other hand, storage of several genotypes in *ex situ* collections coming from different locations gives extra safety for the conservation of a certain species. Over time, the traditional methods of storing the genetic resources as seeds and plant collections contributed to the conservation of genetic fond of the world. Nowadays, the advanced modern methods in plant biotechnologies are applied on a larger scale for the conservation of plant genetic resources. Worth mentioning that these modern methods do not exclude the traditional *in situ* and *ex situ* conservations, but are complementary methods of conservation. A wide variety of modern methods, such are *in vitro* biotechnologies, genome analyzing molecular methods, cryopreservation protocols, or immunologic diagnosis methods are used now for the characterization of plant collections, as well as for the propagation and multiplication, diseases indexing, conservation, distribution or plant material exchange.

Sterilizing the plant material with Domestos detergent (a variant) was less efficient (18.18 – 63.64 % sterile explants) than the mercuric chloride (b variant) (37.93 – 84 % sterile explants), and the combined method (c variant) gave the best results (60 – 92 % sterile explants), without affecting viability (Fig. 5).

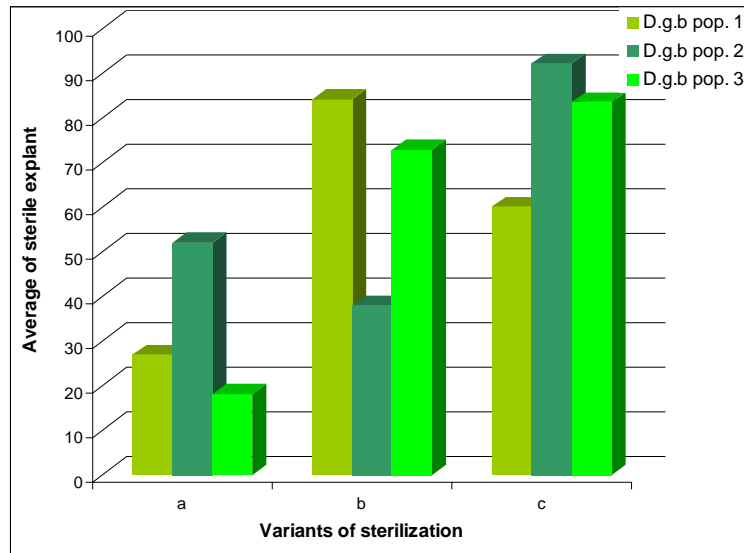


Fig. 5. The average of sterile explants of *Dianthus g. subsp. banaticus* still viable after sterilization (**a**= Domestos detergent, **b**=HgCl₂, **c**= Domestos detergent + HgCl₂).

The observations done at 30 and 60 days from the transfer on D2 (MS medium with vitamins, with 20 g/l sucrose, BAP 1mg/l, NAA 0.1 mg/l), suggest that if after 30 days the populations multiplied relatively uniform, after 60 days there is an obvious difference between the 3 populations (Fig. 6). The number of neoshoots of the somaclones form the same individual have a wide variability, the multiplication rate oscillating between 10 and 135 neoshoots/inoculum within the same individual (Fig. 7). We mention that somaclones are represented by explants coming from the same vitroplantlet.

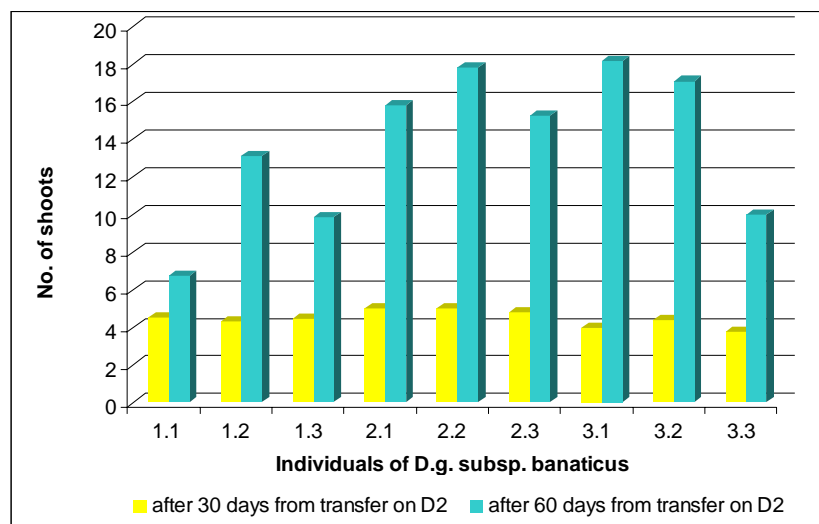


Fig. 6. The multiplication of *D. g. subsp. banaticus* individuals on D2 medium (1.1, 1.2, 1.3= ind. pop. 1; 2.1, 2.2, 2.3=ind. pop.2; 3.1, 3.2, 3.3=ind. pop.3).

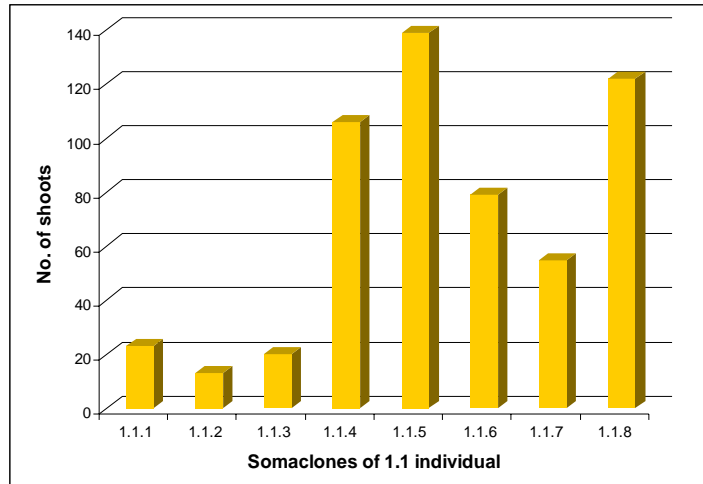


Fig. 7. The multiplication of somaclones of 1.1 individual, at 60 days from the transfer on D2 medium

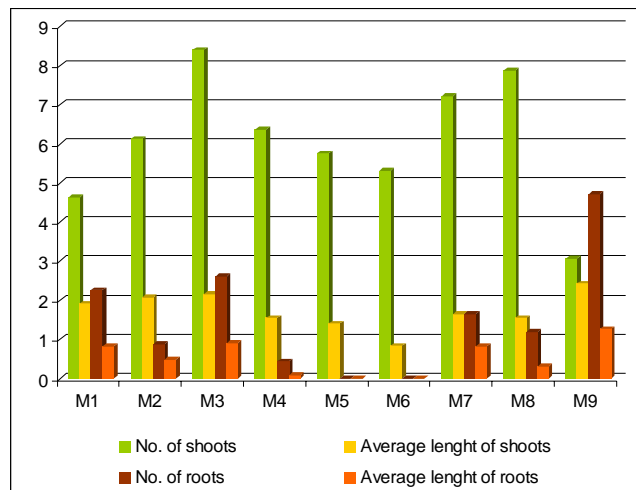


Fig. 8. The influence of culture medium on *D. g.* subsp. *banaticus* multiplication, at 40 days from inoculation: **M1**=K 0.1mg/l, **M2**=K 1mg/l, **M3**=BAP 0.1mg/l, **M4**=BAP 1mg/l, **M5**=2iP 10mg/l, **M6**=2iP 15mg/l, **M7**=TDZ 0.01mg/l, **M8**=TDZ 0.05mg/l, **M9**=MS.

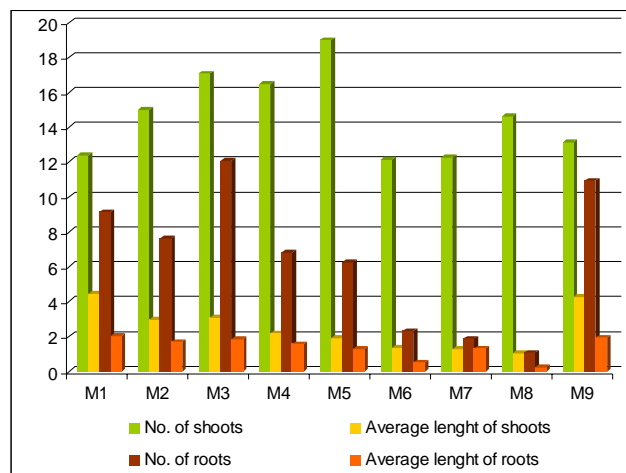


Fig. 9. The influence of culture medium on *D. g.* subsp. *banaticus* multiplication, at 110 days from inoculation: **M1**=K 0.1mg/l, **M2**=K 1mg/l, **M3**=BAP 0.1mg/l, **M4**=BAP 1mg/l, **M5**=2iP 10mg/l, **M6**=2iP 15mg/l, **M7**=TDZ 0.01mg/l, **M8**=TDZ 0.05mg/l, **M9**=MS.

It is well known that sometimes culture media without phytohormones stimulate rhizogenesis, and many times allow satisfactory *in vitro* multiplication. In this respect, an experiment was set up to compare the evolution of explants on culture media with phytohormones (Tabs. 2 and 3) and on MS medium without phytohormones.

The average of shoots number after 40 days from inoculation (Fig. 8) on the 8 culture media with hormones and one without hormones, shows that in case of cytokinines K (M1 and M2) and TDZ (M7 and M8) a higher concentration of the hormone in the culture medium (1mg/l K and 0.05mg/l TDZ respectively) causes a better multiplication rate than BAP (M3 and M4) and 2iP (M5 and M6), which gives a better multiplication at a lower concentration (0.1 mg/l BAP and 15mg/l 2iP respectively). Concerning the medium without hormones (M9), it causes the formation of a lower number of shoots, but the inocula have numerous roots.

After 110 days from inoculation, the same ratio is preserved as after 40 days, regarding the multiplication according to the used hormonal balance, but the best development is present to the inocula on the M1, M2, M3 and M9 culture media (Fig. 9), for the shoots number and their length, as well as for the roots number, which are numerous on these media.

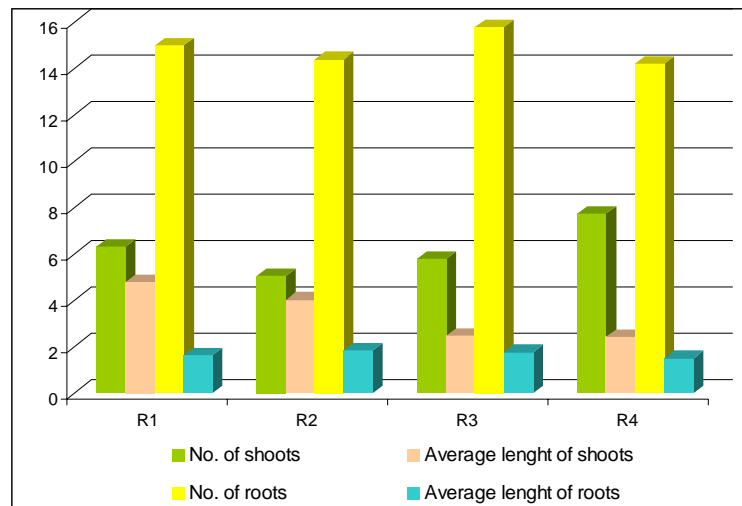


Fig. 10. The influence of culture medium on *D. g. subsp. banaticus* rhizogenesis after 70 days from inoculation: **R1**=MS, **R2**=NAA 0.1mg/l, **R3**=NAA 1mg/l, **R4**=IAA 1mg/l

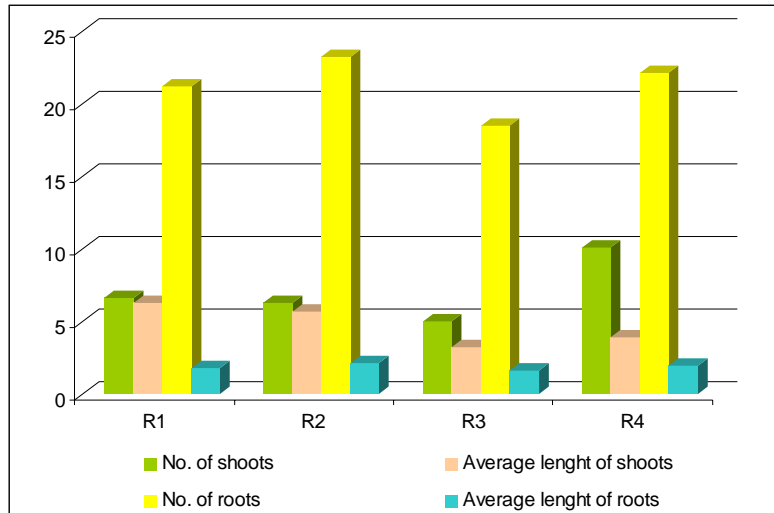


Fig. 11. The influence of culture medium on *D. g. subsp. banaticus* rhizogenesis after 140 days from inoculation: **R1**=MS, **R2**=NAA 0.1mg/l, **R3**=NAA 1mg/l, **R4**=IAA 1mg/l

Although sometimes the roots formed *in vitro* culture are fragile and can be easily destroyed when acclimatized, is preferable to obtain them *in vitro* for easing the transfer from an environment with 100% humidity, to one with a lower humidity. For *in vitro* inducing roots formation a culture medium with hormonal balance favoring auxines is used. In our study, for detection of an optimum culture medium for *D. giganteus* subsp. *banaticus* roots formation, we used two types of auxines (NAA and IAA), in different concentrations, but also a culture medium without hormones.

Observations regarding rhizogenesis were done at 70 and 140 days from inoculation and the number and length of roots were monitored, and also the shoots. At 70 days from inoculation (Fig. 10) a good development of the roots an all the culture media can be observed. At 140 days (Fig. 11) a balance is maintained regarding the number of roots/inoculum, in all variants.

Kovac (1995) when studying *in vitro* multiplication of *Dianthus arenarius* subsp. *bohemicus* obtained the best multiplication rate and rhizogenesis on medium without hormones with 15% sucrose and ½ MS. Also, Dace et al., (2004) obtained a good multiplication rate and rhizogenesis of several species on MS medium without hormones. In our study, we obtained comparable results with the ones obtained on media with auxines, on media without phytohormones (R1=MS medium).

c. *In vitro* photoautotrophic culture and microscopy

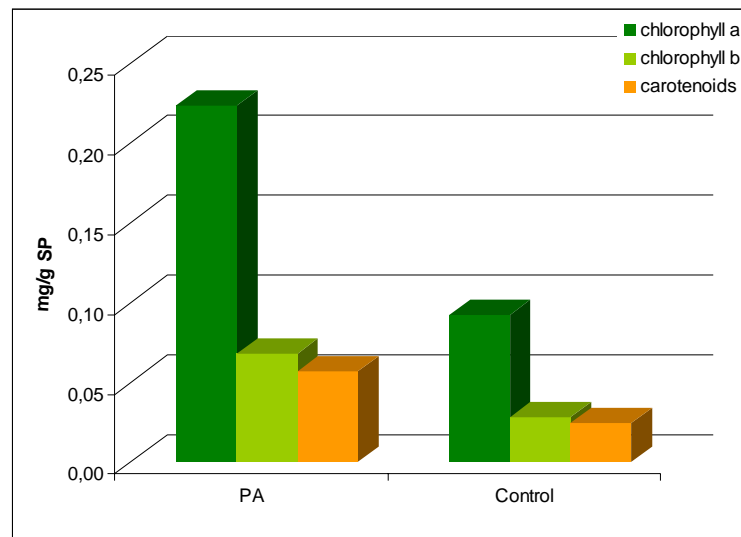


Fig. 12. The content of assimilatory pigments from *D. g. subsp. banaticus* in the two types of *in vitro* cultures

In our experiments of inducing *in vitro* photoautotrophic cultures, the content of assimilatory pigments in the two variants of cultures (photoautotrophic and control) was monitored, at 60 days from the initiation of cultures. The results show (Fig. 12) a higher content in plants from photoautotrophic culture than *in vitro* classical culture. A similar aspect is revealed by Cristea et al., 1999 in chrysanthemum.

In the structural (Fig. 13) and ultrastructural (Fig. 14) investigations, numerous changes in the leaf blade can be observed, in the three types of cultures. These changes, induced by *in vitro* culture, are reversible. After a period of maintenance the plants in photoautotrophic culture, these changes reduce.

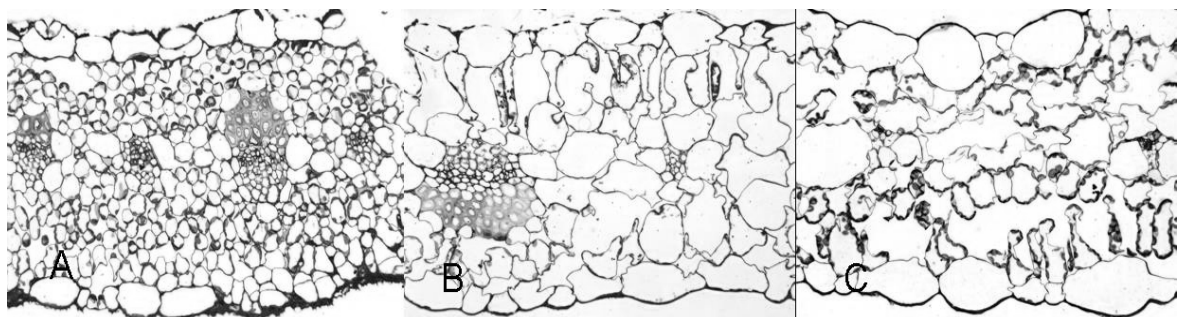


Fig. 13. Structural aspects in the leaves of plants from the three types of cultures: A-natural habitat, B-*in vitro* classical culture, C-photoautotrophic culture

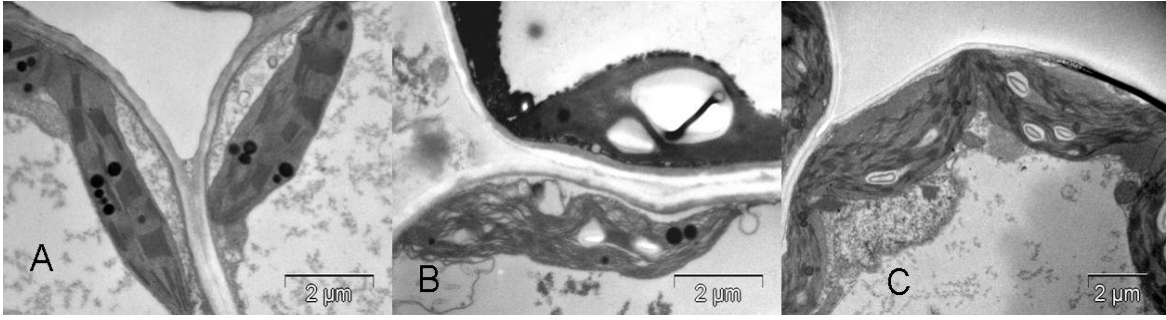


Fig. 14. Ultrastructure of chloroplast in the leaves of plants from the three different cultures: A-natural habitat, B-*in vitro* classical culture, C-photoautotrophic culture

d. Cryopreservation

In case of cryopreservation experiments, we can notice (Fig. 15) a reducing of regeneration capacity along with increasing the sucrose concentration. When apices were cryopreserved, the highest values regarding viability and regeneration capacity were recorded in the treatment with 0.5 M sucrose solution. The duration of treatment with vitrification solution had different effects. Thus, a reducing of regeneration capacity along with increasing the incubation time in vitrification solution for control apices (- liquid nitrogen) was observed. In the case of cryopreserved apices (+ liquid nitrogen) the highest percentage of regeneration was recorded at an incubation time of 15-20 minutes in the vitrification solution (Fig. 16). The first signs of apices regeneration after freezing in liquid nitrogen were observed after 7-15 days (Fig. 17).

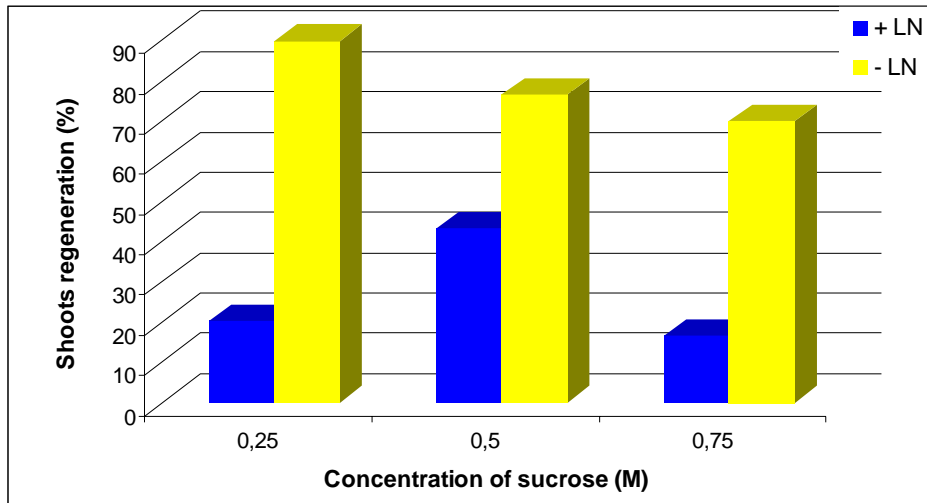


Fig. 15. The influence of sucrose concentration on the regeneration capacity of control and cryopreserved apices

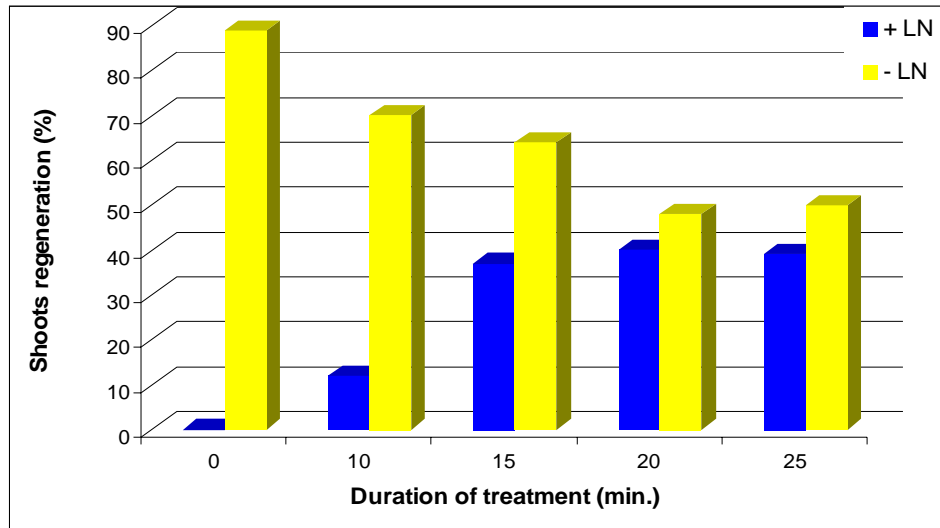


Fig. 16. The influence of treatment duration with vitrification solution on regeneration of control and cryopreserved stem apices

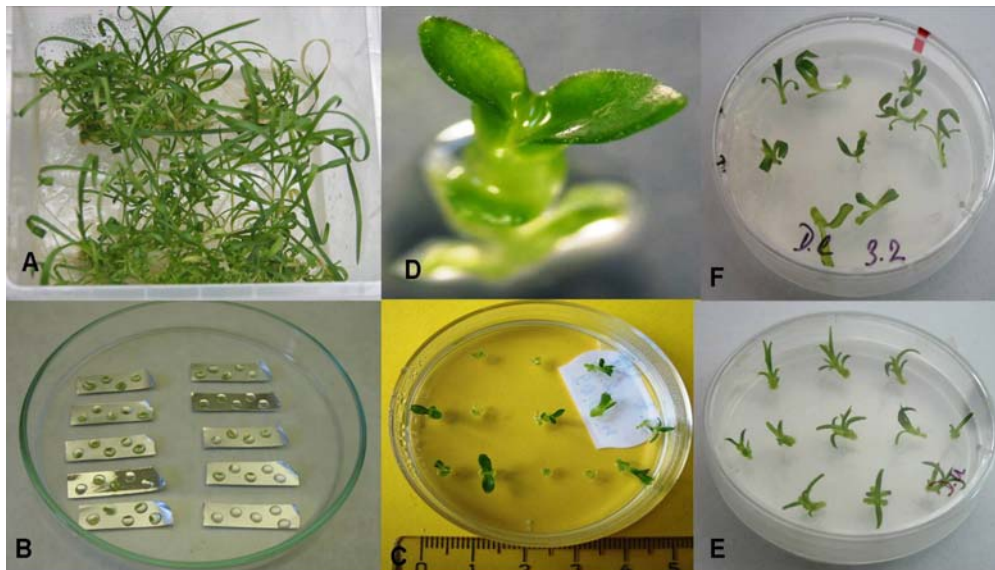


Fig. 17. Aspects regarding *in vitro* multiplication of apices after cryopreservation and their transfer on regeneration media (A- *in vitro* plants; B-meristems in PVS2 drops; C, E, F- plants on regeneration medium; D-apex at 7 days after liquifying)

3. 3 Study of somaclonal variability in *Dianthus giganteus* subsp. *banaticus*

The somaclonal variability is defined as variability induced by different variants of cells and tissue cultures (Bairu et al., 2010). Since 1958, when Braun did the first remarks regarding the somaclonal variability, it remained an obstacle for the cells and tissue cultures. The growth and development of plants by micropropagation is an asexuate process, which should not produce variability (Bairu et al., 2010), but it should theoretically be a cloning process (Larkin, 1998).

The somaclonal variability induced by *in vitro* culture is a process used in the genetic manipulation of some cultures, but when we refer to the conservation of a certain taxon and the opportunity to reintroduce it in the original sites, the somaclonal variability is not a wanted process, and the plants that suffered such a mutation can not be used for the ecologic reconstruction of a site.

Regarding the conservation of some phytogeographical important, endemic or endangered taxa, it is necessary to do investigations by molecular biology methods, which complete data obtained by conventional techniques. Thus, before the implementation of a conservation program it is necessary to identify the biologic material to be conserved. The next step is knowing the genetic variability in taxon populations to be conserved and verifying the somaclonal variability at the end of *ex situ* conservation, before reintroduction in the wild (Butiuc-Keul, 2006).

DNA analysis with ISSR (BC809 primer) shows a high genetic polymorphism of *Dianthus giganteus* subsp. *banaticus* individuals (Fig. 18). By DNA amplification with MS-DINCARACC primer (SSR marker) two fragments were identified – the first (of 200 bp) is present to all the studied individuals whatever the population, and the second (of approximately 75 bp) scarcely present in some individuals from populations 2 and 3 (Figs. 19, 20).

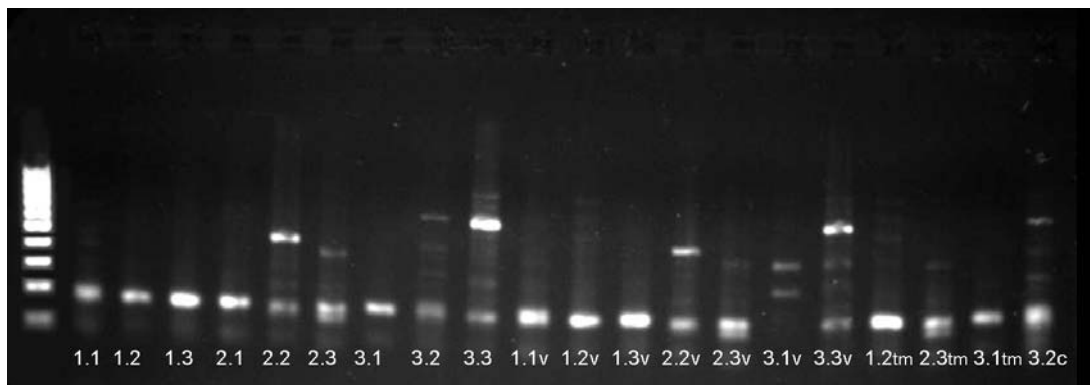


Fig. 18. Banding pattern obtained with BC 809 primer in *D. g. banaticus* individuals from the natural habitat and *in vitro* conserved, on medium term, and cryopreservation

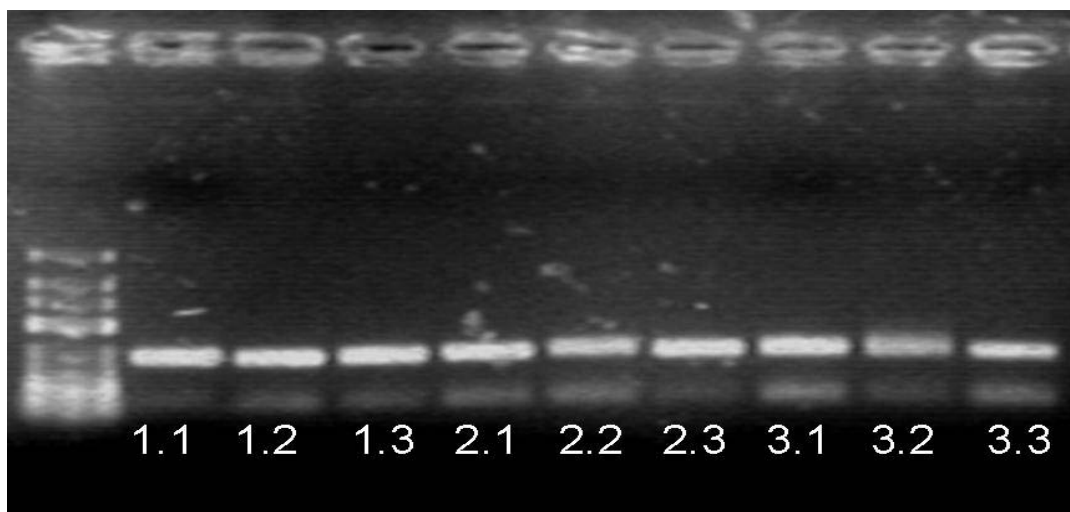


Fig. 19. Banding pattern obtained with MS-DINCARACC primer in *D. g. banaticus* individuals from the natural habitat and *in vitro* conserved, on medium term, and cryopreservation

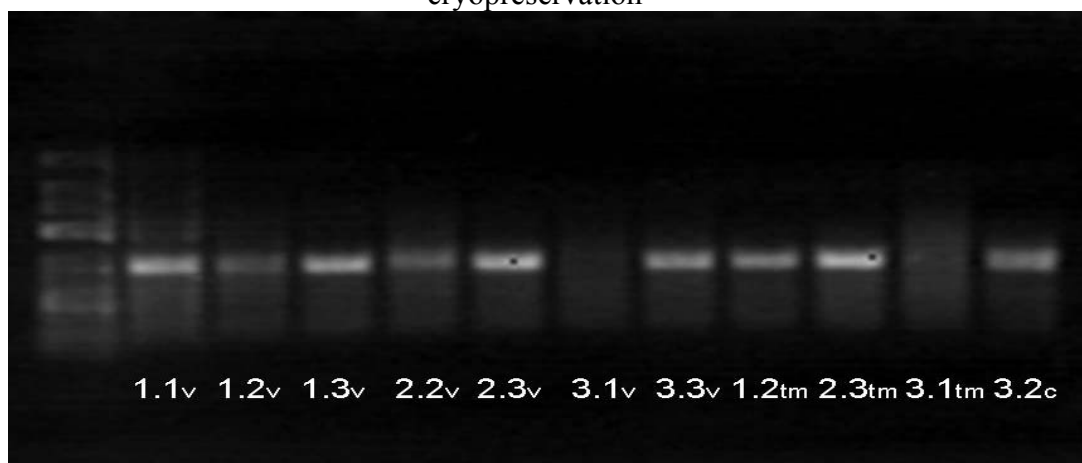


Fig. 20. Banding pattern obtained with MS-DINCARACC primer in *D. g. banaticus* individuals from the natural habitat and *in vitro* conserved, on medium term, and cryopreservation

Molecular techniques are useful instruments in analyzing the somaclonal variability in micropropagated plants. The molecular markers are able to identify certain fragments from the DNA sequence, associated with genome parts, and the comparisons are usually done regarding the presence/absence of these fragments (Gostimsky et al., 2005). The studies regarding the assessment of somaclonal variability in *D. giganteus* subsp. *banaticus* show some changes concerning the absence/presence of certain fragments in some individuals, but how much is affected the genome is still a subject to be discussed.

Similar studies done in *Dictyospermum ovalifolium* (Chandrika et al., 2008) also show the appearance of variability in micropropagated plants by the absence/presence of certain fragments, while in *Nothapodytes foetida* (Chandrika et al., 2010) the banding

patterns obtained with each primer were uniform with those of original plant, but anyway they were both acclimatized and reintroduced in the natural habitat.

Thus, our study shows that somaclonal variability took place in the studied taxon, but how important is this is still to be studied.

Conclusions

1. According to the eco-coenotic study of *Dianthus giganteus* subsp. *banaticus* taxon we found that it is present in 10 plant communities, but it best grows in the ambiance of *Agrostio tenuis-Festucetum rupicolae*, *Arrhenatheretum elatioris*, *Thymo pannonici-Chrysopogonetum grylli* and *Festucetum rupicolae* phytocoenoses.

Besides the literature data, which mentions only 4 plant communities with *D. giganteus* subsp. *banaticus*, our study emphasizes larger eco-coenotic amplitude of this important taxon.

Comparing the ecologic needs of this taxon with the dominant note of the 10 plant communities, a good correlation is seen with *Agrostio tenuis-Festucetum rupicolae* plant community, where the number of *D. giganteus* subsp. *banaticus* cushions and the AD values are higher.

Besides the theoretical value, this study has also a practical importance, because it suggests the possibility to rehabilitate *D. giganteus* subsp. *banaticus* populations in eco-coenotic ambiance offered by *Agrostio-Danthonietum provincialis*, *Rhinantho rumelici-Brometum erecti* and *Thymo comosi-Galietum albi* plant communities

2. *Ex situ* conservation was done outdoor on a special rock in the Botanical Garden, as well as *in vitro*.

In case of *in vitro* cultivation, we recommend the use of a combined method with several sterilization agents (e.g. Domestos, HgCl₂), which complementary act on different pathogenic agents, for obtaining a good rate of sterilization of explants.

Comparing the influence of culture medium on *in vitro* multiplication and rhizogenesis in *D. giganteus* subsp. *banaticus* we observed that by using a MS culture medium without phytohormones we obtained comparable results with those from culture media with phytohormones. Thus, when needed plant material for an eventual repopulation purpose, it is recommended to use this type of culture medium, reducing the cost price, as well as the probability of somaclonal variability in the obtained plants.

3. Following the investigations done by means of light microscopy, it was observed that numerous changes appear in the leaf blade, among the three types of cultures. These changes induced by *in vitro* culture are reversible. After a period of maintenance the plants in photoautotrophic culture, these changes reduce, as it can be seen in the similarities that appear among the sections of plants leaves from the wild and those from photoautotrophic culture.

The ultrastructural investigations confirm the results obtained in light microscopy. The images obtained for the leaves coming from plants in photoautotrophic culture show a very similar situation to that of the control, in that the changes described in leaflets of plants from vitroculture do not appear anymore or they are minor and insignificant.

Photoautotrophic cultures may replace *in vitro* classical cultures or may constitute a phase before vitroplantlets acclimatization, this phase playing the role to prepare plants for an autotrophic nutrition, to develop the photosynthetic apparatus, which is less developed in plants from *in vitro* classical culture.

4. The cryopreservation studies show that the use of dropped vitrification method was efficient in the conservation of *D. giganteus* subsp. *banaticus* taxon, obtaining a good regeneration rate after preserving probes in liquid nitrogen. For better results, we recommend that meristems to be treated with 0.5M sucrose solution, for 24 h, followed by a treatment with vitrification solution for 20-25 minutes.

5. Study of somaclonal variability in the taxon show the appearance of some changes regarding the number of fragments and their molecular weights, among the individuals from the natural habitat and the cryopreserved ones.

6. Our thesis responds to the 3 objectives of the National Strategy for Biodiversity Conservation by the theme, approach and conclusions.

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