IN VITRO INTRODUCTION OF *Dianthus trifasciculatus* KIT ssp. *parviflorus* AS *EX SITU* PRESERVATION METHOD

HOLOBIUC Irina, CATANĂ Rodica, VOICHIŢĂ Carmen, HELEPCIUC Florenţa

Abstract. Taking into account the strong decrease of plant diversity owing to the anthropically modified environment and climatic changes, it is necessary to adopt integrative measures performed both *in situ* and *ex situ* to ensure plant preservation. Among *ex situ* methods, besides classical ones, plant biotechnologies bring an important contribution for biodiversity preservation. *In vitro* cultures can provide plant material for natural habitats repopulation and restoration of wild populations, and also for *ex situ* conservation during different period. *D. trifasciculatus* KIT ssp. *parviflorus* is a critically endangered taxon and it is common to the South of Romania and the North of Bulgaria. This taxon is endangered owing to anthropically influences and invasive species. *In vitro* cultures were started from explants collected from a plant from the Botanical Garden "Al. Borza" Cluj Napoca. Different media variants added with growth factors (cytokinins and auxins) were tested for primary cultures induction, for optimization of the regeneration and for rooting. An efficient multiplication protocol was established for this taxon useful for conservation purpose, which can be extended for the multiplication of several genotypes from natural populations.

Keywords: *in vitro*, conservation, direct morphogenesis, *Dianthus trifasciculatus* KIT ssp. *parviflorus.*

Rezumat. Introducerea *in vitro* ca metodă de conservare *ex situ* a taxonului *Dianthus trifasciculatus* KIT ssp. *parviflorus.* Ţinând cont de diminuarea dramatică a biodiversităţii vegetale, în condiţiile antropizării mediului, a schimbărilor climatice globale, este necesară adoptarea de măsuri integrate care să asigure conservarea speciilor de plante atât *in situ* cât şi *ex situ*. Între metodele *ex situ*, pe lângă cele clasice, biotehnologiile vegetale aduc un aport important la conservarea biodiversităţii plantelor. Culturile *in vitro* pot asigura material biologic pentru repopularea habitatelor şi refacerea populaţiilor naturale, cât şi pentru conservarea *ex situ* pe diferite durate de timp. *D. trifasciculatus* KIT. ssp. *parviflorus* este un taxon critic periclitat, comun regiunii sudice a României şi nordului Bulgariei. Acesta este amenitaţ ca urmare a antropizării habitatelor şi a pătrunderii speciilor invazive. Culturile *in vitro* au fost iniţiate de la un individ existent în colecţia Grădinii Botanice „Al. Borza" Cluj Napoca. Diferite variante de medii adiţionate cu factori de creştere (citokinine şi auxine) au foste testate pentru inducerea culturilor primare, pentru optimizarea regenerării şi pentru înrădăcinare. A fost elaborat un protocol eficient de multiplicare, util din punct de vedere conservativ care poate fi exins pentru multiplicarea mai multor genotipuri din populaţiile naturale.

Cuvinte cheie: *in vitro*, conservare, morfogeneză directă, *D. trifasciculatus* KIT ssp. *parviflorus.*

INTRODUCTION

In Romanian Flora, from 3795 *Thracheophyta* species and subspecies, ~30 % are threatened (DIHORU & NEGREAN, 2009). 110 taxa are considered European or/and Globally threatened (OPREA, 2005). The causes of the disappearance of the species could be anthropic and/or natural. The increased rhythm of disappearance of plant species owing to anthropic pressures and global climatic changes imposes integrative measures of conservation performed both *in situ* and also *ex situ*. Among *ex situ* conservation measures, besides the traditional approaches (in seeds banks, field collection or botanical gardens), the biotechnological approaches can significantly contribute to the preservation of plant biodiversity.

The **Global Strategy for Plant Conservation** (2011-2020) claimed that at least 75% of the threatened plant species to be preserved in *ex situ* collections, preferably in the country of origin, and at least 20% to be available for recovery and restoration programs (Target 8).

In Romania, the **National Strategy and the Action Plan for Biodiversity** (SNPAB) refers to measures concerning the biodiversity conservation between 2011 and 2020, with 10 objectives. Among these, *ex situ* preservation has an important place sustained by scientific research and elaboration of technologies applicable in the case of threatened and/or endemic plants.

In vitro cultures can ensure in short time, in reduced space-and with relative low labour cost, plant material for recovery of wild populations or for preservation during different period of time. Another important advantage is that it can provide healthy plant material non-exposed to biotic and abiotic stress factors.

Taking into account the duration and methods used *in vitro*, the preservation can be done during short-term - through active cultures multiplication during 1-3 months or medium-term, using growth retardation protocols, during several months or years. On the other hand, long-term preservation involves the storage in liquid nitrogen of apexes, meristems or somatic embryos, which suffered different treatments to counteract the formation of the ice inside the cells.

In fact, short-term and medium term preservation can supply plant material both for *in situ* reintroduction and also for long-term preservation of threatened plant taxa.

At international level, several authors sustained the role of biotechnology in plant biodiversity conservation (BENSON, 1999; ENGELMAN, 2010; REED et al., 2011; GONZALEZ-BENITO & MARTIN, 2011; BUNN et al., 2011).

The taxon taken into study, *D. trifasciculatus* ssp. *parviflorus* is cited in the Red Book of Vascular Plants from Romania, being common to the South of Romania and the North of Bulgaria. It is a perennial, amphimictic, allogame

taxon, growing on neutral, poor substrate. It is a critically endangered taxon (DIHORU & NEGREAN, 2009), fact that justifies the *in vitro* conservation studies. The reduction of the plant areal owing to agricultural practices and invasive species determines this taxon to become threatened (DIHORU & NEGREAN, 2009).

MATERIAL AND METHODS

For *in vitro* cultures initiation, we used explants collected from a plant cultivated in the "Al. Borza" Botanical Garden, Cluj-Napoca.

In vitro primary culture was started from single node stem fragments. Sterilization was made in four steps:

I. washing in running tap water II. short immersion in 70^0C ethylic alcohol for 30 second III. sterilization with mercuric chloride 0.1% for 2 times x 5 minutes; IV. three times washing with sterile distilled water. The rate of sterilized explants was recorded after one week.

As the first plant material used to start the *in vitro* cultures was limited, at the beginning, just few media variants were tested for the primary cultures induction (Table 1).

The culture media tested were based on Murashige-Skoog formula (MURASHIGE & SKOOG, 1962) modified by adding 30 g/l sucrose and B5 vitamins (GAMBORG et al., 1968) and growth factors in different combinations for the regeneration process in primary and secondary cultures (Table 1 and 2).

To stimulate rhizogenesis, there were used media based on MS formula modified through the reduction of macro- and microelements (R1-R5) and sucrose content - R5 variant (Table 3).

The media were solidified with 0.8% Plant agar and pH was adjusted at 5.75-5.8 using 1N KOH before autoclaving.

Different growth factors were added into the regeneration media to sustain the *in vitro* developmental processes: cytokinins as benzyl aminopurine - BA, kinetin - K, adenine - Ad, zeatin - Zea and auxins as alpha-naphthyl acetic acid - NAA, 2,4 - dichlorophenoxyacetic acid - 2,4-D. Glutamine was also used as supplement.

Table 1. The media cultures tested for the primary in vitro cultures.

Components		**Media variants**				
		M1	**M2**	**M3**	**M4**	**M5**
Macroelements		MS	MS	MS	MS	MS
Microelements		MS	MS	MS	MS	MS
Growth factors (mg/l)	**BAP**	**1**	**1**	**1**	**1**	**-**
	Kin	**1**	**1**	**1**	**1**	**-**
	Zea					**2**
	Ad	**-**	**-**	**50**	**-**	**-**
	NAA	**0.2**	**0.2**	**0.2**	**-**	**0.2**
	2,4- D	**-**	**-**	**-**	**0.2**	**-**
Others Compounds (g/l)	**Glut**	**-**	**0.2**	**-**	**-**	**-**

Table 2. The media used in the second cultures for improving the regeneration rate.

Components		**Variants**								
		M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**
Macroelements		MS	MS 1/2	MS 1/2	MS	MS	MS	MS	MS	MS
Microelements		MS	MS 1/2	MS 1/2	MS	MS	MS	MS	MS	MS
Vitamins		B5	B5	B5	B5	B5	B5	B5	B5	B5
Growth factors (mg/l)	**BAP**	**0.1**	**0.1**	**0.1**	**0.1**	**-**	**-**	**1**	**1**	**-**
	Kin	**-**	**-**	**-**	**-**	**0.1**	**0.1**	**1**	**1**	**-**
	Zea				**-**				**-**	**2**
	GA_3	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**0.5**	**-**
	NAA	**-**	**0.01**	**0.01**		**0.01**	**-**	**-**	**0.2**	**0.2**
	2,4- D	**-**	**-**		**0.01**	**-**	**0.01**	**0.2**	**-**	**-**
Other compounds (g/l)	**Glut**	**-**	**-**	**0.2**	**-**	**-**	**-**	**-**	**-**	**-**

Legend: MS - Murashige & Skoog medium (MURASHIGE & SKOOG, 1962); B5 Gamborg - vitamins (GAMBORG et al., 1968); BAP - benzyl aminopurine; Kin- kinetin; Zea - zeatin, Ad - adenine; NAA - α-naphthyl acetic acid; 2.4-D - 2.4 - dichlorophenoxyacetic acid; Glut - glutamine, GA_3- gibberellic acid.

The evaluation of the regeneration in the primary culture was done through registering the number of developed shoots/inoculum, without statistical repetitions because of the small quantity of initial explants. For every medium variant, there were cultured 2 Petri dishes with 3 stem fragments as explants.

In the second culture cycle, for improving the regeneration rate, double node shoots fragments collected from the primary regenerative cultures were used as explants and cultured on nine media variants. Five explants in 3 repetitions were cultured for every medium.

The mean number of regenerated shoots was registered after 30 days and statistically analysed using ANOVA test.

The media with the best results in the primary cultures, containing normal levels of growth factors (M7-M9) were compared concerning the efficiency of the regeneration process with variants containing reduced levels of growth factors (M1-M6) and low content of macro- and micro-elements (M2-M3) (Table 2).

The cultures were maintained at 2000 lux illumination and 16/8 photoperiod and 25^0C temperature.

For rooting, several minimal R1-R5 media were tested (Table 3). Medium MS added with 30 g/l sucrose was considered as control (R6). R1-R5 variants consisted in modified MS formula by reducing salts at ½ and ¼ and sucrose used at 20 g/l and 10 g/l. Additional shoots formation and roots development were evaluated as mean number/initial explant after 40 days of culture.

Table 3. Minimal media tested for rooting.

Components	Media variants					
	R1	**R2**	**R3**	**R4**	**R5**	**R6**
Macroelements	MS 1/2	MS 1/2	MS 1/2	MS 1/4	MS 1/4	MS
Microelements	MS 1/2	MS 1/2	MS 1/2	MS 1/4	MS 1/4	MS
NAA	-	-	0.01	-	-	-
AC	-	500	-	-	-	-
Sucrose (g/l)	20	20	20	20	10	30

Legend: MS- Murashige & Skoog medium, AC-active charcoal, NAA- alpha naphthyl acetic acid.

RESULTS AND DISCUSSIONS

There were already made several studies concerning *in vitro* multiplication for conservative purpose in different *Dianthus* threatened taxa (ZĂPÂRŢAN, 1995; BUTIUC-KEUL & DELIU, 2000; PĂUNESCU & HOLOBIUC, 2003; CRISTEA et al., 2002, 2004, 2006, 2010; MICLĂUŞ et al., 2003; HOLOBIUC et al. 2004-2005; MARCU et al., 2006; HOLOBIUC & BLÎNDU, 2006; HOLOBIUC et al., 2009; HOLOBIUC et al., 2010; JARDA et al., 2010; RADOJEVIČ et al., 2010, POP & PAMFIL, 2011). In almost all studied taxa, the main way of *in vitro* regeneration was direct morphogenesis.

The sterilization rate of the explants consisting in single node stem fragments was 80%.

D. trifasciculatus ssp. *parviflorus* showed a positive *in vitro* reactivity in the primary cultures (Table 4).

The cytokinin dominance in the culture media allowed a good regeneration through direct morphogenesis occurred at the level of lateral meristems.

The best responses in the primary culture was registered after one month of culture on the variants M2 (supplemented with BAP/K/NAA and glutamine), M4 (supplemented with BAP/K/2,4-D) (Fig. 3b) and M5 (supplemented with zeatin and NAA) (Fig. 3a).

On the M2 and M4 variants rooting of the shoots occurred, while in the case of M5, no roots formed, but callus structure arose at the end of the shoots.

Table 4. *In vitro* response in primary cultures.

Media variants used	Cytokinins/ auxins combination	The response after one month of culture	The response after 2 months
		The mean number of shoots/inoculum	The number of shoots/ initial explant
M1	BAP/K /NAA	4	15-20
M2	BAP/K/ANA +glutamine	3.3	15-40
M3	BAP/K/ Adenine/NAA	6	20-25
M4	BAP/K/2,4-D	9	20-30
M5	Zeatin/ NAA	15	20-25

Despite of good regeneration rate, in the first 30 days on medium supplemented with zeatin, the efficiency of the regeneration was not better than those registered on other variants added with usual cytokinins such us BAP alone or associated with kinetin. Besides this, the callus induction at the end of the shoots and no rooting process is unfavourable for conservative and multiplication purpose.

After two months of primary cultures, the regenerative response was significantly improved. The number of shoots were increased reaching a maximum of 40/ initial explant (M2) (Table 4). Medium variant supplemented both with BAP and kinetin at 1mg/l and 2.4-D at 0.2 mg/l had also a beneficial effect concerning the regeneration (Fig. 3c).

Similar results concerning *in vitro* regeneration was reported by us in other related taxa belonging to *Dianthus* genus (HOLOBIUC et al., 2006, 2009, 2010), the ratio cytokinins/ auxins 10:1 being most beneficial for a majority of *Dianthus* studied.

In the case o f *D. giganteus* ssp. *banaticus,* JARDA et al. (2010) reported that the combination of BAP at 1mg/l and NAA 1 mg/l (1/1 ratio) and sucrose at 20 g/l was suitable for primary culture establishment. For the second multiplication cycle, the level of NAA was decreased at 0.1 mg/l.

In the ratio 10/1 between BAP and NAA was also found to be optimally for shoots regeneration.

RADOJEVIČ et al., (2010) reported in *D. giganteus* ssp. *croaticus* and *D. ciliatus* ssp. *dalmaticus* that medium MS supplemented with BAP 1 mg/l, NAA 1 mg/l and IBA 1.5 mg/l was favourable for multiplication starting from stem segment cultures. In the case of media added with high level of auxins (5mg/l 2.4-D) combined with others growth factors, callus formation was described, but this indirect way of regeneration is not preferable for threatened or endemic taxa because it can generate variability.

In this experiment, the conversion into plants of possible proembryogenic structures developed from callus did not occurred in the case of *D. giganteus* ssp.*croaticus* and *D. ciliatus* ssp. *dalmaticus.*

POP & PAMFIL (2011) also studied *in vitro* regeneration in *D. spiculifolius, D. henteri* and *D. giganteus* ssp. *banaticus*, the cultures being started from aseptic germinated seeds. They concluded that the ratio 10/1 of BAP/ NAA determined the best results concerning multiple shoots formation after 30 and 120 days of culture in all studied taxa, but the authors faced with some problems with the rooting of the regenerated shoots on the media tested, the results being not satisfactory enough. Due to this aspect, the acclimatization of the plants was not efficient.

In our study, in the second culture cycle, starting from shoots derived from primary cultures as inocula, it was compared the efficiency of the regeneration using culture media with low content of salts and growth factors (diluted ten times) with complex media with normal levels of cytokinins and auxins (Fig. 1).

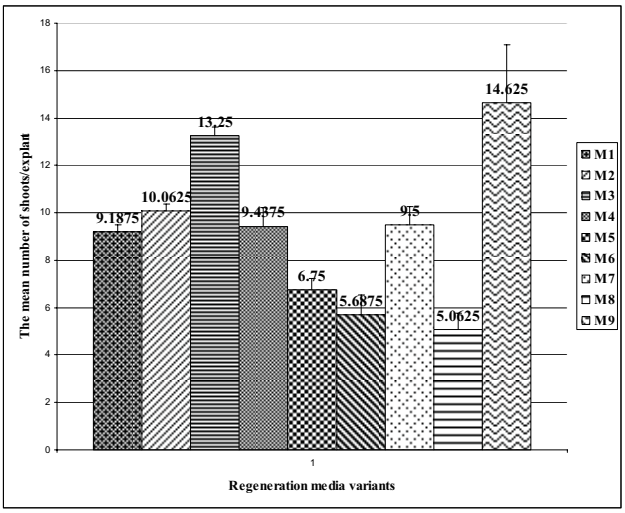

Figure 1. The mean number of regenerants/ explant in the second experiment in *D. trifasciculatus* ssp. *parviflorus* after 30 days of culture.

In this experiment*, D. trifasciculatus* ssp. *parviflorus* proved to regenerate in good conditions both on normal levels and also on reduced concentrations of growth factors.

The simpler variants with low content of growth factors (M1-M6) also proved to sustain the regeneration process through direct shoot formation with positive results.

M5 medium added with kinetin and NAA at low levels and keeping 10/1 ratio determined the development of white callus at the end of explants similar to zeatin/NAA added medium at normal concentrations.

Among the variants with reduced growth factors content, M3 variant characterized by the presence of glutamine as supplementary source of nitrogen, favoured the best results concerning regeneration (Fig. 3b). The mean number of shoots/ explants was very close to the result obtained in the presence of Zeatin (an expensive growth factor), the last one conducting to vitrification of the shoots (Fig. 3a).

Table 5. Observations concerning the *in vitro* response in the second culture cycle on optimised regeneration variants (M1-M9).

Variants	***Observation***
M1	Good regenerative response, shoots of 1-2 nodes, roots formation
M2	Good regeneration, reduced growth at one node, without roots, slow callusing process
M3	Very good regeneration, without rooting
M4	Good regeneration, shoots elongation at 2-3 nodes, some shoots necrosis
M5	Medium regeneration rate, absent rhizogenesis, white callus formation at the end of the shoots
M6	Good regeneration, shoots elongation at 2-3 nodes, good vigour, without roots
M7	Good regeneration, shoots elongation at 2-3 nodes, good vigour, without roots
M8	Medium regeneration rate, shoots elongation at 2-3 nodes, good vigour, without roots
M9	Very good regeneration rate, vitrified shoots without roots

For rhizogenesis process, all tested minimal media (R1-R5) sustained root formation (Fig. 2a).

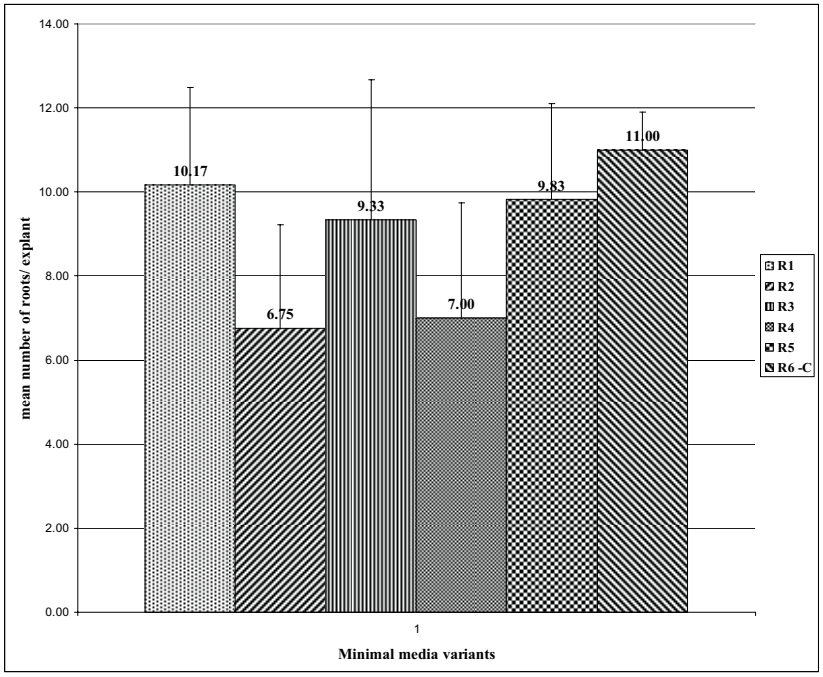

Figure 2a. Mean number of roots/ inoculum on minimal culture media after 40 days.

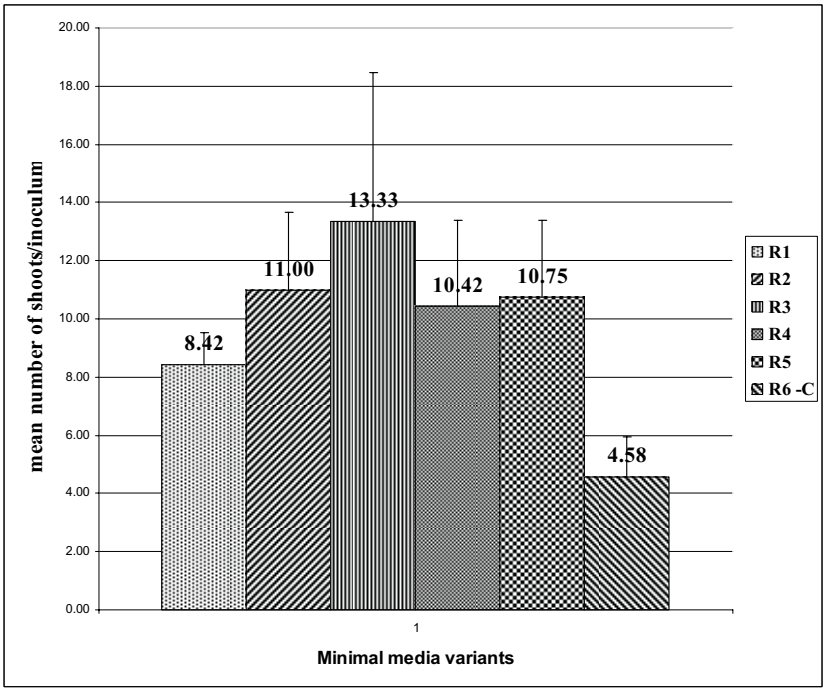

Figure 2b. Mean number of shoots/ inoculum on minimal culture media after 40 days.

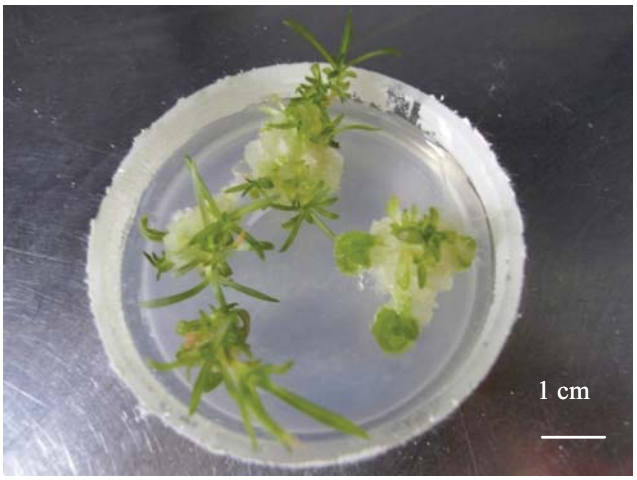

Figure 3a. Shoots regeneration after 1 month on Zeatin and NAA supplemented medium in primary culture (original photo).

Figure 3b. Direct morphogenesis on variant supplemented with BAP 0.1 mg/l, NAA 0.01 mg/l 1 mg/l after 2 months of culture (original photo).

Figure 3c. Shoots formation on variant supplemented with BAP, Kinetin 1 mg/land 2.4-D 0.1 mg/l after 2 months (original photo).

Figure 3d. Explants rooting and supplementary shoots formation on minimal R1 medium (original photo).

Figure 3e. Explants rooting and supplementary shoots formation on minimal R5 medium (original photo).

Figure 3f. *Ex vitro* acclimatization of regenerated plants on pot (original photo).

R1, R3, R5 variants showed results comparable with the control R6 (MS medium added with 30 g/l sucrose), concerning the rooting, the first ones being cheaper to use.

Besides root formation, on these minimal culture media, lateral shooting occurred with different rates (Fig. 2b). The number of developed shoots was comparable to those obtained in the case of optimized culture media (Fig. 3d, e).

The best result concerning lateral shoots formation on minimal media, was registered on R3 variant with macro- and microelements reduced at half, 20 g/l sucrose and 0.1 mg/l NAA.

For economic reason, it is recommended to use for primary culture establishment, regeneration media added with cytokinins and auxins at 10/1 ration, being preferred BAP and NAA or BAP, Kinetin and NAA or 2,4-D and subsequently the transfer on minimal culture media for rooting and supplementary shooting.

The *ex vitro* acclimatization of the well-grown and rooted plants involved:

- the cultivation in pots using sterilized substrate made from ground/ Perlite in 1:1 ratio;
- the maintenance of the humidity at 80% through coverage with plastic bags during the first 4 days and subsequently the gradual opening for 30 minutes daily during two weeks.

The survival rate of acclimatized plants (Fig. 3f) was about 80% using this protocol.

CONCLUSIONS

The studied taxon showed a good *in vitro* reaction, a regeneration protocol being established for conservative purpose.

This method can be useful for the multiplication and *ex situ* preservation of several individuals (genotypes) to extent the variability of the preserved germplasm.

It is recommended to use for the primary culture induction a complex medium variant based on normal MS formula, added with 30/l sucrose and as cytokinin BAP associated with NAA and glutamine 0.2 g/l, keeping 10/1 ratio between the growth factors.

For the secondary multiplication and rooting, it can be used minimal variants based on MS formula reduced at half and added with 0.01 mg/l NAA, medium which sustains both rooting and development of supplementary lateral shoots.

In two months of culture, it can be regenerated a lot of clonally plants which can be used for further studies, acclimatized and cultured in outdoor *ex situ* collections.

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Holobiuc Irina
Institute of Biology Bucharest, Romanian Academy,
296 Splaiul Independenţei,
060031 Bucharest, P.O. Box 56-53, Romania.
E-mail: irina.holobiuc@ibiol.ro

Voichiţă Carmen
Institute of Biology Bucharest, Romanian Academy,
296 Splaiul Independenţei,
060031 Bucharest, P.O. Box 56-53, Romania.
E-mail: carmen.voichita@ibiol.ro

Catană Rodica
Institute of Biology Bucharest, Romanian Academy,
296 Splaiul Independenţei,
060031 Bucharest, P.O. Box 56-53, Romania.
E-mail: catanarodica@yahoo.com

Helepciuc Florenţa
Institute of Biology Bucharest, Romanian Academy,
296 Splaiul Independenţei,
060031 Bucharest, P.O. Box 56-53, Romania.
E-mail: florentaelenah@gmail.com

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