MEDIUM-TERM PRESERVATION OF *Dianthus trifasciculatus* Kit ssp. *parviflorus* THROUGH MINIMAL CULTURES

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Abstract. The species taking in our study, *Dianthus trifasciculatus* ssp. *parviflorus* is a critically endangered taxon, *in vitro* studies being useful for *ex situ* preservation purpose. Owing to *in vitro* rapid growth and proliferation, it is important to establish a medium-term conservation protocol based on minimal cultures. Several factors were tested concerning the ability to reduce the growth of tissues cultures and to maintain regeneration capacity. Mannitol and PEG 4000 were proven to be the most suitable for the establishment of minimal cultures. Regenerants from three years old minimal cultures maintained in the presence of mannitol were analysed concerning isoenzymes spectrum for peroxidase, esterases, alkaline phosphatases and catalases. Biochemical analyses have shown that peroxidases (POX) and catalases (CAT) are sensitive to the culture conditions (the maintenance in presence of osmolytes). The treatment with 6% mannitol influenced the intensity of the bands analysed in the case of rooted plants even after 2 months of culture in the absence of any osmolyte. The analysis of esterases and alkaline phosphatases spectra has shown the regenerants identity.

Keywords: *Dianthus trifasciculatus* Kit ssp. *parviflorus*, medium-term, minimal cultures, mannitol, isoenzymes spectra.

Rezumat. Conservarea pe termen mediu a speciei *Dianthus trifasciculatus* Kit ssp. *parviflorus* în culturi minimale. Specia studiată de noi**,** *D. trifasciculatus* ssp. *parviflorus* este un taxon critic periclitat, studiile *in vitro* fiind utile pentru conservarea *ex situ*. Datorită creşterii şi proliferării rapide este important să se stabilească un protocol de conservare pe termen mediu bazat pe culturi minimale. Mai mulţi factori au fost testaţi pentru abilitatea de a reduce creşterea culturilor de ţesuturi şi de a menţine capacitatea regenerativă. Manitolul şi PEG 4000 s-au dovedit a fi cei mai potriviţi pentru stabilirea culturilor minimale. Regeneranţi proveniţi din culturi minimale de trei ani, menţinute in prezenţa manitolului au fost analizaţi pentu spectrul peroxidazelor, esterazelor, fosfatazei alcaline şi catalazelor. Analiza biochimică a aratat că peroxidazele şi catalazele sunt sensibilie a condiţiile de cultură (menţinerea în prezenţa osmoliţilor). Tratamentul cu 6 % manitol influenţează intensitatea benzilor analizate în cazul plantelor înrădăcinate chiar după 2 luni de cultură în absenţa oricărui osmolit. Analiza spectrelor esterazei şi fosfatazei alcaline au arătat indentitatea regeneranţilor.

Cuvinte cheie: *Dianthus trifasciculatus* Kit ssp. *parviflorus*, termen-mediu, culturi minimale, manitol, spectrele izoenzimatice.

INTRODUCTION

Biotechnology has proved its role for the plant biodiversity preservation, several results being obtained in the case of cultivated plants, but also in the case of wild species and especially the threatened ones (BENSON, 1999; HOLOBIUC, 2006; SARASAN et al., 2006; ENGELMAN, 2010; REED et al., 2011; BUNN et al., 2011). As a huge progress occurred in this area of research and many achievements are permanently reported, it is very important that the results (technologies) to be recognized as useful and to be effectively integrated in the conservation programs (BENSON, 1999).

Biotechnology alternative based on *in vitro* cultures has not to substitute the classical successful methods, but to complement them or to insure a reliable alternative in the case of threatened taxa with reproduction problems, low variability and/ or small populations.

Tissue cultures ensure the preservation during different periods of time, independent of climate changes, pests, in limited spaces. On the other hand, owing to the requirement of qualified staff, expenses for establishment and maintenance of cultures and energy consumption, it is necessary to evaluate if the proposed aim of the conservation is appropriate to be achieved using *in vitro* cultures. For this reason, it is important to establish optimized, reproducible and low costing technologies, which can be easily implemented in practice.

Depending on the durations and methods used, three levels of conservation are available for *ex situ* preservation: 1) short- term conservation: primary tissues cultures established for multiplication purpose, for temporary storage of plant germplasm, for repopulation of the natural habitats and for international exchanges, 2) medium-term conservation, during months and years, based on growth retardation of the tissues cultures 3) long-term conservation, relied on cryopreservation procedures, which allow the maintenance on an indefinite period of time.

Medium-term conservation is based on the maintenance of tissue cultures in the presence of different restrictive factors. The so called "slow growth" method is the result of the reduction of growth rate and multiplication (WHITERS, 1987). Minimal cultures are obtained owing to this growth reduction and development through the modulation of different factors (LYNCH, 1999). The interval of transfers can be prolonged, the cultures vessels can be smaller and medium consumption and handling are also limited.

Medium-term preservation based on minimal cultures was extensively used especially in the case of cultivated species (MALAURIE, 1993; WYSOTSKAYA, 1994; REED & CHANG, 1997; REED, 1999; SARKAR & NAIK, 1998, SARKAR et al., 1999; GOPAL et al., 2002; CHA-UM et al., 2006) and also of wild threatened plants (REED et al., 2011).

In this field, different methods have already been reported based on the modulation of the physical factor as: temperature, light, oxygen content (MULLIN & SCHLEGEL 1976; WESCOTT, 1981; REED, 1993; MORIGUGHI et al., 1995, CACHIŢĂ & HALMAGY, 1997; SARKAR & NAIK, 1998), a combination of several factors (REED, 1999; NEGASH et al., 2001) or chemical factors as: nutrient reduction (low mineral or sucrose concentrations) (NG & NG, 1991,

MALAURIE et al., 1993), encapsulation in alginate (WITHERS, 1991; ENGELMANN, 1991; MALAURIE et al., 1998, HOLOBIUC et al., 2009b), addition of osmotic active compounds (STARITSKY et al., 1986; NG & NG, 1991; CONSTANTINOVICI, 1996; GOLDMIRZAIE & TOLEDO, 1999, NAGATOME et al., 2000; HOLOBIUC et al., 2009a; 2010) or the use of the growth retardants as flurprimidol or ancymidol (JARRET et al., 1991; SWARUP et al., 2001) or growth inhibitors as Abscisic acid (HALMAGY et al., 2001).

For the *ex situ* preservation of threatened plant taxa, slow growth methods or minimal cultures represent convenient alternatives and they can contribute to the establishment of *in vitro* collections (CONSTANTINOVICI, 1996; PENCE, 1999; HOLOBIUC et al., 2010).

In Romania, several achievements concerning the establishment of primary cultures and multiplication based on short-term protocols in *Dianthus* threatened species were obtained (ZĂPARȚAN, 1995; CRISTEA et al., 2002; MICLĂUS et al., 2003; CRISTEA et al., 2004; MARCU et al., 2006; HOLOBIUC et al., 2006; HOLOBIUC et al., 2009c; HOLOBIUC et al., 2010b; HOLOBIUC et al., 2013; CRISTEA et al., 2013; JARDA et al., 2014).

In the case of medium-term and long- term conservation of threatened *Dianthus* taxa, just few studies were reported in Romania (HOLOBIUC et al., 2009a, b; HOLOBIUC et al., 2010; CATANĂ et al., 2010; JARDA et al., 2011).

The species analysed in our study, *D. trifasciculatus* ssp. *parviflorus* is a critically endangered taxon (DIHORU & NEGREAN, 2009*), in vitro* studies being useful for *ex situ* preservation purpose.

In this taxon, an optimized protocol for short-term preservation and multiplication have already been reported (HOLOBIUC et al., 2013), but as all *Dianthus* species, this plant has an *in vitro* rapid growth and multiplication rate, for this reason being important to establish a medium-term conservation protocol based on minimal cultures.

MATERIALS AND METHODS

For medium-term cultures initiation, we used as explants double node shoots fragments collected from the primary regenerative cultures initiated from one individual. They were inoculated on different media variants based on MURASHIGE & SKOOG formula (1962), added with Gamborg vitamins (GAMBORG et al., 1968) and supplemented with different compounds. Several factors were tested for the induction of minimal cultures as osmolytes (PEG 4000, mannitol, sucrose) and plant growth regulators as Abscisic acid (ABA) and Jasmonic acid (JA) (Table 1).

Table 1. Media composition used for the induction of minimal cultures in *D. trifasciculatus* ssp. *parviflorus*.

Composition		**Media variants**							
		M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**
Macroelements		MS	MS	MS	MS	MS	MS	MS	MS
Microelements		MS	MS	MS	MS	MS	MS	MS	MS
Complex B Vitamins		B 5	B5	B5	B5	B5	B5	B5	B5
Plant regulators (mg/l)	**Abscisic acid**	**-**	**-**	**-**	**-**	**-**	**-**	**20**	**-**
	Jasmonic acid	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**3**
Other compounds (g/l)	**Mannitol**	**-**	**30**	**60**	**-**	**-**	**-**	**-**	**-**
	PEG 4000	**-**	**-**	**-**	**-**	**-**	**60**	**-**	**-**
	Sucrose	30	30	30	**60**	**90**	30	30	30
Agar (g/l)		8	8	8	8	8	8	8	8

For each treatment, there were cultured 5 explants/ glass vessel in 3 repetitions. All the cultures were maintained in the growth chamber at 2000 lux illumination and 16/8 photoperiod and 25^0C temperature.

The cultures were evaluated using 2 parameters: the maximum length of the developed shoots/ initial explant (in mm) and the mean number of regenerants/ initial explant scored after different time intervals (40, 80 and 120 days, respectively) to characterize the behaviour and main step of establishment of minimal cultures in this taxon.

Graphic values are expressed as mean values ±SD. The data were statistically analysed using Daniel's XL Toolbox version 6.52. One-way analysis of variance (ANOVA) was applied to calculate the statistical significance at $p<0.05$. Multiple Tukey Comparison test was also used to compare the means at 5% probability level.

The minimal cultures with the best response concerning the growth reduction and regeneration capacity were maintained in the *Dianthus* taxa collection established by us during several years.

From three years old minimal cultures, maintained on 3% and 6% mannitol added media, were regenerated plants after their rooting on IBA (1mg/l) supplemented culture medium. These regenerants were biochemically analysed concerning isoenzymes spectrum for peroxidase, esterases, alkaline phosphatases and catalases to check if the preservation in medium-term culture during several years influenced their stability.

Biochemical analyses of the regenerants from medium-term cultures: rooted plants obtained from 3 years long-term cultures maintained in the presence of mannitol 3 % and 6 % as factor which reduces the growth, but also compatible with the maintenance of regeneration ability and long survival of the cultures were analysed concerning the pattern of several isoenzymes as peroxidases, esterases, catalases and alkaline phosphatases.

Two samples were collected from every 5 jars (I-V) and the number of individuals analysed were 1, 1', 2, 2',3, 3', 4, 4' and 5, 5'. The samples from the first jar (1 an1') derived from medium-term cultures maintained in the presence of 6% mannitol, the others from cultures maintained in the presence of 3% mannitol.

Preparation of total protein extract.

The plant material was ground with quartz sand for obtaining the homogenate of the total protein extract. The extraction of enzymes was carried out in 0.05M phosphate buffer pH 7.2mM EDTA, PVP 4%, at 4°C for 4h. After centrifugation at 18000 rpm, for 20 min, the supernatant was used for electrophoresis. It was prepared the running polyacrylamide gel 7% (for catalases) and 10 % (for esterases, phosphatases and peroxidases) and stacking gel; as buffer we used 0.05M Tris-Gly, pH 8.3. The running marker was bromophenol blue.

Isozymes electrophoretic spectra.

a) For peroxidases detection, a solution of benzidine in acetate buffer and H_2O_2 as substrate was used. The bands were stained in brown (WANG & WANG, 1989). A solution of benzidine in acetate buffer and H_2O_2 was used as substrate.

b) For esterases detection, the substrate α, β–naphthyl acetate and Fast Blue RR dissolved in phosphate buffer 0.1 M, pH=6.5 were used. The bands were stained in red-violet. (BACH, 1989, a modified method).

c) For catalases, 0.003% H_2O_2 prepared in 0.01 M phosphate buffer, pH=7, 2% $K_3(Fe(CN)_6)$ and 2 % $FeCl_3$ were used as substrate. The bands were stained in yellow with a background green-blue (IORDĂCHESCU & DUMITRU, 1988).

d) For phosphatases alkaline, as substrate Na α –naphthyl phosphate, 0.5M $MgCl_2$ and 0.25 M $MnCl_2$ and Fast Blue RR in Tris-citrate, pH=8,3 was used. The bands were stained in brown.

RESULTS AND DISCUSSIONS

As a condition to establish medium-term cultures as *ex situ* preservation tool is necessary to have a reproducible and affordable protocol, which can help to minimize the growth and also to maintain the regeneration ability with a reduction of the interval of cultures transfers.

Using double node-stem fragments as explants, several variants of media were tested concerning the growth and regeneration response of the explants to check which is the most appropriate for our aim to maintain in collection as minimal cultures. The parameters scored after different time intervals (40, 80 and 120 days) showed significant differences concerning the growth and regeneration between M1 (control) and the other variants M2-M7 (Tables 2 and 3).

Concerning the growth reduction, the first parameter scored by us (the maximum growth or length of the shoots developed on the initial explant), comparing to M1 medium, on almost all variant tested (excepting M8 added with Jasmonic acid) was registered a growth retardation effect (Fig. 1).

In the case of the osmolytes addition in the culture media, the reduction of growth was pronounced in the first months. This effect of growth limitation was maintained during the time as effect of osmotic stress imposed by mannitol, PEG or sucrose.

In the case of use of PEG 4000 at the level tested (6%), the reduction of growth registered after 40 days was weaker compared to mannitol and even to sucrose added media. The rooting process occurred more slowly compared to the variants with mannitol.

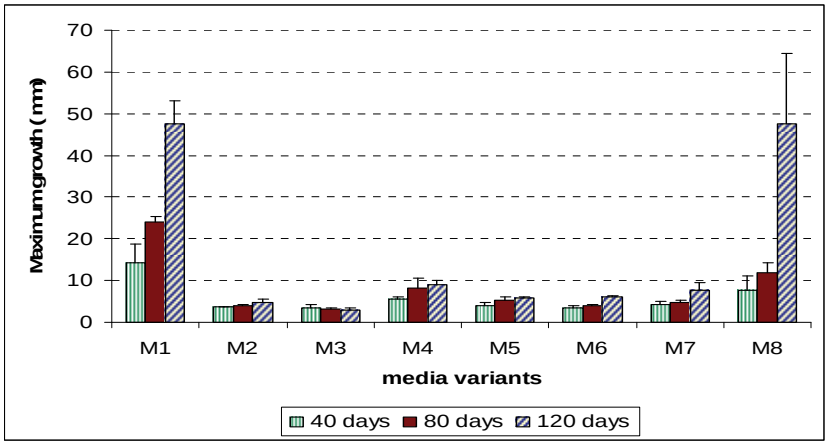

Figure 1. Maximum growth of the shoots registered in minimal cultures of *D. trifasciculatus* ssp. *parviflorus* after different interval of time.

Table 2. Mean values and standard deviation for maximum growth of shoots/explant registered on different media variants after 40, 80 and 120 days.

Variants analysed	M1	M2	M3	M4	M5	M6	M7	M8
40 days	14,33±4,34*	3,6±0,2 *	3,46±0,7*	5,47±0,5*	3,87±0,83*	3,4±0,52*	4,27±0,64*	7,53±3,51*
80 days	24±1,44*	3,87±0,23*	3,2±0,34*	8,27±2,41*	5,27±0,75*	3,93±0,3*	4,73±0,57*	12±2,39*
120 days	47,67±5,47*	4,67±0,94*	3±0,4*	8,87±1,28*	5,8±0,2*	6±0,34*	7,73±1,81*	47,52±16,95

Legend: Values are means of 3 replicates with standard error. * showed that the values are significantly different from the M1 (control) by the Tukey test at ($P<0.05$).

Cultures performed on variants M2, M3, M6 showed a growth limitation similarly after 40 days.

The level of 6% sucrose was less limiting concerning the growth of shoots comparing to the higher level (9%) concerning the growth of shoots. After increasing the period of the culture, sucrose in excess proved to have a toxic effect inducing shoots death, despite of some inductor effect concerning the regeneration, aspect more evident after 3 months of exposure. Some already developed shoots degenerated and some new developed. After 120 days, a generally reduction of the growth of the shoots was noticed in the cultures maintained on the variants M2-M7 (Fig. 1).

Abscisic acid presence (20 mg/l) determined the arrest of the growth and absence of regeneration; the lateral meristem of explants could be maintained in a latent stage during 2-3 months.

Jasmonic acid added in variant M8 determined a good vigour of plants, but did not reduce the growth, which was similarly to the control, allowing the development of 1-2 lateral shoots.

Concerning the induction and the maintenance of the regenerative process, the best values were registered in the presence of mannitol 3% and PEG 4000 at 6% level starting from the first month of culture (Fig. 2).

The regeneration capacity scored as mean number of shoots/ explant showed that after a first slow inhibitory effect after the initiation of the cultures in the first month, lately a stimulatory effect was detected; the variant added with mannitol at 3% level induced the best response after 80 days (Fig. 3a) and 120 days of maintenance, the number of regenerants increasing in time despite their reduced growth. The initial explants degenerated, but new regenerants developed starting form lateral meristems as primary origin.

Also the presence of 6% mannitol had an inductor effect on the regeneration process, with the best rate after 120 days. The stress effect was overcome later, in the second month on this variant, the degeneration of the first regenerants occurred, but the extension of the maintenance in the same conditions allowed the development of a large number of small propagules (regenerants) (Fig. 2) after 120 days.

In the case of PEG 4000, the effect concerning the reduction of the growth was not so strong, but also stimulated the regeneration process after a delay of the morphogenesis in the first month, a resuscitation of the process appeared subsequently.

In the first phase of the establishment of the medium-term cultures, PEG presence inhibited the rooting process, but in the second month of culture, the primary stress effect was overcome by the plants adaptation mechanisms and rhizogenesis process was also induced (Figs. 3e, 3c).

The addition of sucrose at 6 and 9% final concentrations as osmotic factors determined the reduction of the growth (Fig. 1) and sustained the regeneration process, especially in the first two months (Fig. 2).

The mean values of the regenerants/explant were lower than mannitol and PEG 4000 - added variants and decreasing in time owing to the degeneration of the neo-formed shoots. Despite of the positive effect observed in the first month, the signs preceding the necrosis and cellular death were correlated to the activation of the synthesis of anthocyanins (Fig. 3d, f). As result, after 120 days, some of the shoots died, but some new buds developed for a short time.

The extension of the duration of the maintenance of the cultures more than 120 days conducted to the conclusion that minimal cultures in this taxon can be optimally performed in the presence of mannitol 3 %, the reduced growth being associated with very good regeneration, results comparable to other *Dianthus* taxa, studied by us (HOLOBIUC et al., 2010).

In the presence of PEG 6%, the regeneration rate decreased comparing to that registered in the presence of mannitol. In the case of sucrose excess, despite the acceptable effect noticed in the first two months, after 120 days, the shoots started to die, after an activation of the synthesis of anthocyanins before this radical response.

In case of the use of Abscisic acid, as growth inhibitor, the level tested by us (20 mg/l) allowed the maintenance of the explant at the initial state for 3 months (the lateral meristems did not grow and not develop lateral shoots). The presence of Jasmonic acid (3 mg/l) had an opposite effect, conducting to similar results concerning the regeneration to the control, but the shoots were better developed.

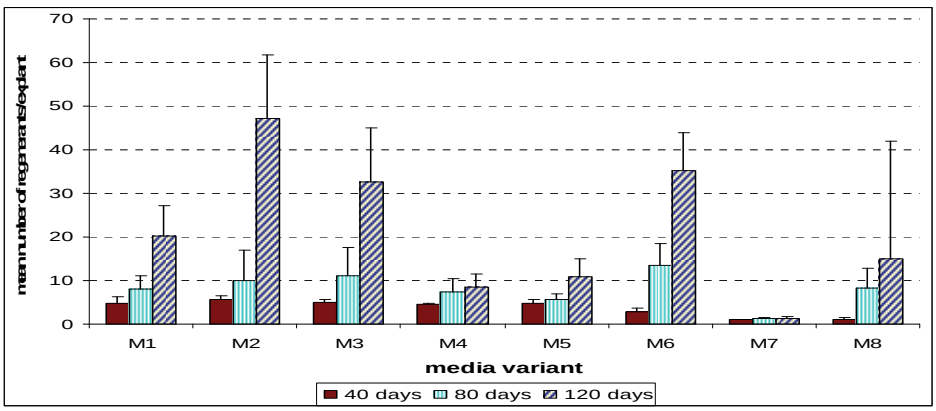

Figure 2. The mean number of shoots/explants registered in the minimal cultures of *D. trifasciculatus* ssp. *parviflorus* after different intervals of time.

Table 3. Mean values and standard deviation for maximum growth of shoots/explant registered on different media variants after 40, 80, and 120 days.

Variants analysed	M1	M2	M3	M4	M5	M6	M7	M8
40 days	4,8±1,4*	5,67±0,9	4,93±0,75	4,67±0,11	4,87±0,8	2,87±0,75	1,07±0,11*	1,08±0,34*
80 days	8±3,04*	9,93±6,98	11±6,53	7,33±3,06	5,6±1,29	13,4±5,06	1,2±0,41*	8,26±4,53
120 days	20,27±6,87*	47,13±14,69	32,66±12,33	8,53±3,04	10,8±4,16	35,13±8,83	1,27±0,45*	15,06±26,81

Values are means of 3 replicates with standard error. * showed that the values are significantly different compared with the M1 (control) by the Tukey test at ($P<0.05$).

Figure 3a. Minimal cultures in *D. trifasciculatus* ssp. *parviflorus* established in presence of 3% mannitol after 80 days (Bar = 1 cm) (original).

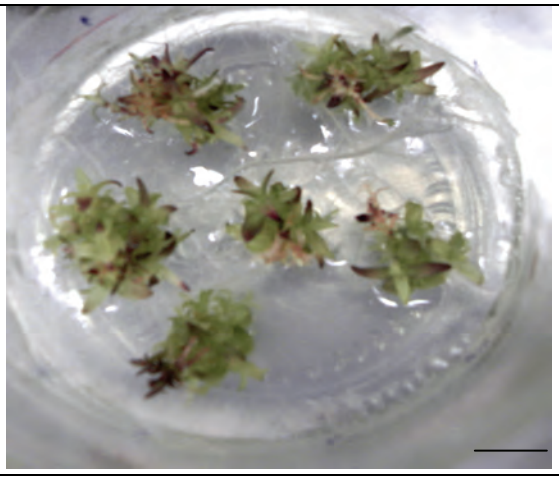

Figure 3b. Minimal cultures with direct morphogenesis and activation of the synthesis of anthocyanins in the presence of 6% mannitol after 80 days (Bar = 1 cm) (original).

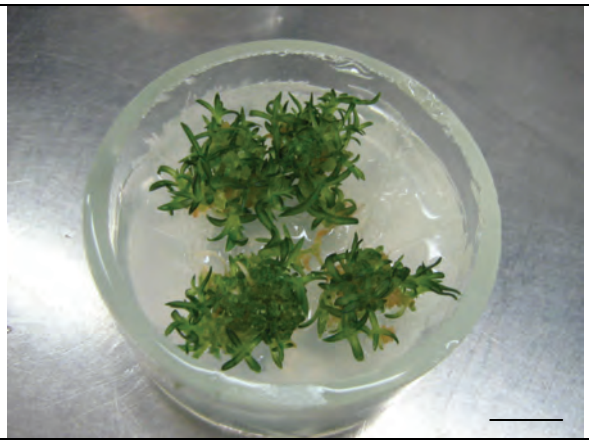

Figure 3c. Regenerative aggregates with limited growth induced in the presence of 6% PEG 4000 after 80 days (Bar = 1cm) (original).

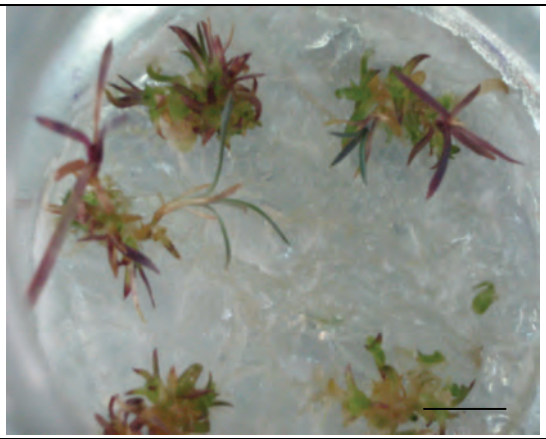

Figure 3d. Morphogenic minimal cultures with active the synthesis of anthocyanins preceding shoots degeneration in the presence of 9% sucrose after 80 days of culture (Bar = 1 cm) (original).

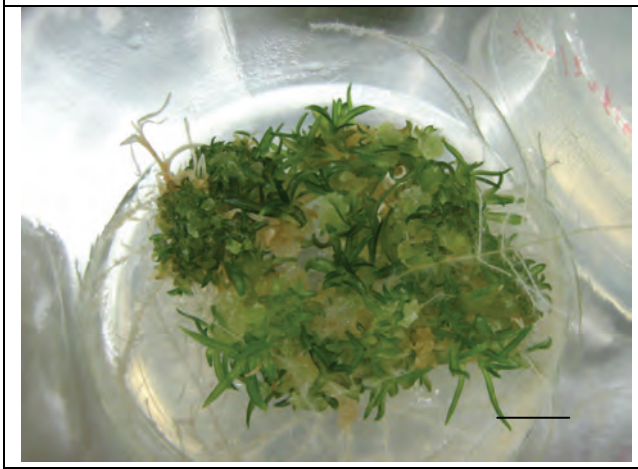

Figure 3e. Morphogenic minimal cultures maintained in the presence of PEG 4000 6% after 6 months (Bar = 1 cm) (original).

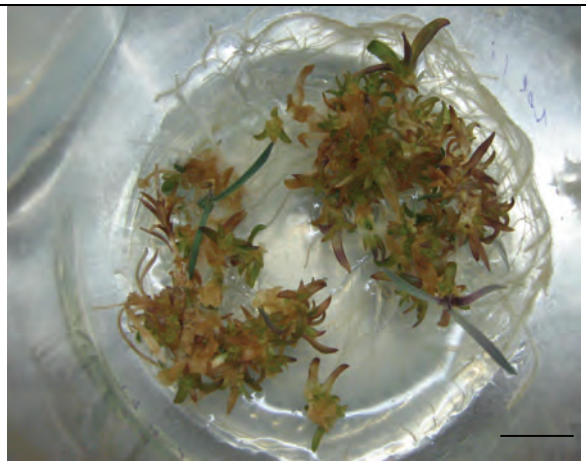

Figure 3f. Minimal cultures maintained in the presence of sucrose 6% during 6 months (Bar = 1 cm) (original).

Biochemical analysis of in vitro regenerated plant material from minimal culture.

The genetic stability of the preserved plant material was usually evaluated in the case of cultivated or medicinal plants *in vitro* regenerated and/or maintained in collections. For this purpose, besides molecular markers, biochemical analysis proved to be useful to study the effect of *in vitro* micropropagation and also for the characterization of medium-term cultures maintained *in vitro*.

Isoenzymes spectra are detected by electrophoresis and specific staining of the bands; a certain configuration of the bands can be detected according to the number of the loci, the homo or heterozygous status and the number of subunits (BUTIUC-KEUL, 2006). In fact, isoenzymes are codified by genes situated on different loci but codifying proteins with the same enzymatic activity.

The results concerning the isoenzymes spectra analysed by us in the case of clones of *D. trifasciculatus* ssp. *parviflorus* regenerated from minimal cultures are shown in figure 4.

Electrophoresis spectra of peroxidases (POX).

Peroxidases electrophoretic spectrum in the case of the samples 2,2', 3,3', 4,4' and 5,5' consisted in 5 bands with some differences of intensity in the case of samples 2,2'-5,5'(more intense)- Fig. 4.

Electrophoresis spectra of POX determined in the case of the samples 1 and 1' originated on medium-term cultures maintained in the presence of a higher level of mannitol (6%) showed a different intensity of bands comparing to the samples originated in medium term cultures maintained on lower level of mannitol (3%).

Alkaline phosphatases electrophoretic spectrum showed a single band in all analysed samples, proving the identity of these clones concerning this character (marker).

Isoesterase spectrum showed also the identity in all samples (clones) analysed, with a more intense expression in the case of samples derived of mannitol 6% cultures.

Peroxidases are inducible and more sensitive to the condition of culture and physiological status of the plants, but isoesterases are more stable, being preferred in some cases to asses genetic diversity or variability.

For **catalase spectrum,** in the samples 1 and l' a low intensity of the bands was detected, but in the case of 3' sample, an intensification of catalase activity and higher intensity of the bands was noticed, meanwhile the clones 2, 2', 3, 4, 4', 5, 5' shown the same pattern of bands.

The use of this biochemical marker is not suitable to check the plants variability, being most susceptible to environmental conditions (the conditions of culture and the developmental stage).

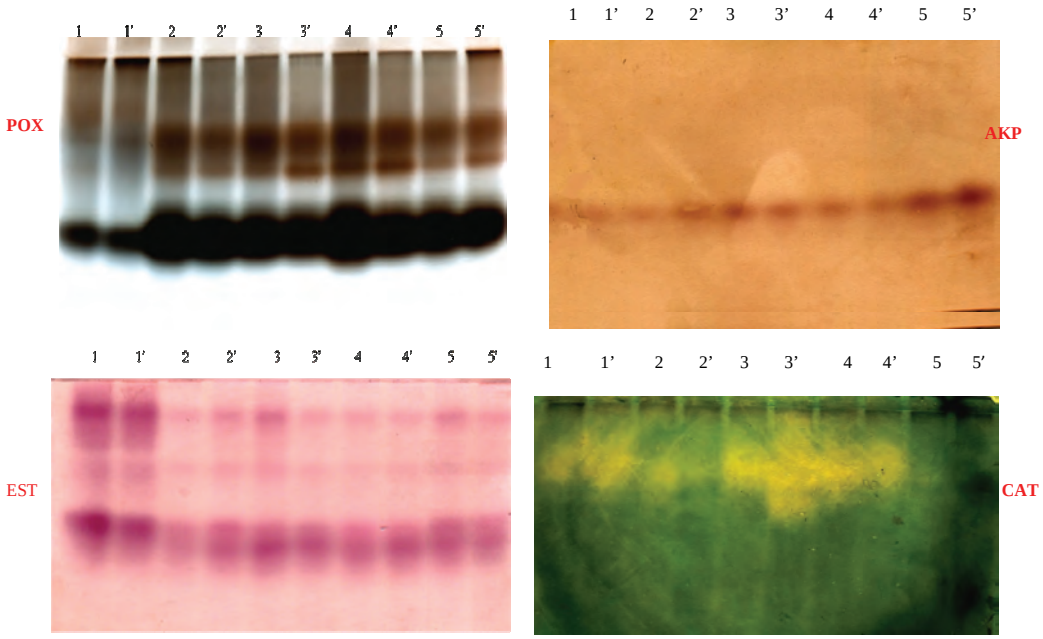

Figure 4. Isoenzymes spectra in the case of regenerants derived from medium-term cultures in *D. trifasciculatus* ssp. *parviflorus* (original).

Isoenzymes were used before to characterize the clones of *in vitro* regenerated *Atropa belladonna* (BUTIUC-KEUL et al., 2005). The aim was to detect lines highly productive of atropine, but isoperoxidases spectrum did not show any differences among the clones.

In the regenerants obtained in the primary cultures of *Arnica montana*, BUTIUC-KEUL & DELIU (2001), reported that isoesterase pattern was the same in all individuals, proved to obtain a clonal propagation.

In sweet potato accessions maintained *in vitro* during 1.6 years, regenerants conserved their isoenzymes profile concerning 5 enzyme systems, showing genetic stability (LAKHANPAUL et al., 1990). KRZAKOWA (1996) reported the use of peroxidase spectrum to detect differences among individuals and populations of *Phragmites australis*.

In the case of two *Dianthus* taxa preserved in medium-term cultures (*D. spiculifolius* and *D. glacialis* ssp.*gelidus*), isoenzymes profiles and activity were used previously to evaluate the cultures maintained in the presence of different levels of mannitol.

The antioxidant enzymes activity and profiles in the first month and after prolonged exposure at osmotic stress (several months) were influenced by the concentration of the osmolytes, the duration of exposure to stress and by the capacity to overcome the stress and to induce developmental processes as morphogenesis and even somatic embryogenesis (HOLOBIUC et al., 2009a; HOLOBIUC et al., 2010). Isoenzymes patterns were correlated to the adaptation to osmotic stress, growth reduction and regeneration process.

CONCLUSIONS

Our studies concerning the induction of minimal cultures in *D. trifasciculatus ssp. parviflorus* proved that taking into account the main purpose, different factors added in culture media can be used to obtain different results: a reduction of growth, but associated to a very high multiplication, a moderate growth and an acceptable multiplication rate or even stopping the development of the explants.

Both mannitol and PEG 4000 can be used for minimal cultures establishment. In case of mannitol, the limitation of growth is stronger and regeneration better. Mannitol at moderate level and PEG 4000 can be used for long-term maintenance of the regenerative cultures. The regeneration rate in the presence of PEG is good, but the rooting of regenerants occurred slowly.

If the aim is just to maintain the explants *per se*, ABA-added medium variant can be used for 2-3 months preservation, the lateral meristems of the explants did not evolve and explants did not root.

On the other hand, the presence of Jasmonic acid in the culture medium improved the plant growth and vigour being suitable to be applied before different selective factors or before acclimatization.

Our results showed that POX and CAT are sensitive to the culture conditions (the previously treatment with osmolytes applied during medium–term cultures). The maintenance of the culture in the presence of 6% mannitol influenced the intensity of the detected bands even after 2 months of culture of regenerated shoots in the absence of any osmolyte.

Esterases and alkaline phosphatases spectra have shown the regenerants plants identity; their origin was the same.

The minimal cultures were successfully established in this taxon and healthy and normal plants can be regenerated even after several years of maintenance.

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