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In vitro conservation under slow growth conditions of two rare plant species from *Caryophyllaceae* family

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Abstract

The purpose of this study was to evaluate the aspects of medium term conservation under slow growth conditions of two rare plant species from *Caryophyllaceae* family: *Gypsophila petraea* (Baumg.) Rchb. and *Dianthus callizonus* Schott et Kotschy. Different methods like reducing carbon source, reducing mineral concentration and temperatures (25°C and 10°C) were tested. The evaluated parameters were represented by the healthy shoots and rhizogenesis. The presence of hiperhidricity was observed. To check the genetic stability of the shoots maintained under slow growth conditions, the biochemical analysis were performed after 6 months. The physical factors (lack of nutrients and temperature) changed the enzymatic spectra, but the biochemical analysis did not show differences between the clones cultured on the same media variant. The 10°C and the reducing of micro- and macronutrients at 1/4 for *G. petraea* and at 1/10 for *D. callizonus* allowed the conservation in optimal parameters for more than 12 months.

Keywords: *in vitro* conservation; slow growth conditions; rare plants; genetic stability; enzymatic spectra.

1. Introduction

According to the World Conservation Union, over 8,000 plant species are threatened with extinction. The are several approaches that can help to save the plant species like: the documentation of global distribution and conservation status of the plant species, the protection of the areas, the education of people about the values of the biodiversity, the existence of national and international legislation etc [1].

In the last decades, the *ex situ* conservation methods have an important role in the conservation of threatened plants. The three main methods used of *ex situ* conservation are represented by: maintaining living plants in cultivation, *in vitro* conservation (short and medium-term conservation) and cryoconservation (long-term conservation).

A proper medium term conservation protocol involves those methods which allow the reduction of plant growth in order to increase the intervals between subcultures [2] and to minimize the use of plant growth regulators, osmolites and other growth retardants [3]. The maintenance of the viability and the genetic stability is the goal of any *in vitro* conservation approach.

Regarding the priority in conservation according to taxonomic categories, the rare plant species have the highest priority induced by their restrictive area [4]. Also, the endemic (restricted in distribution) to small sites or require specialized habitats are listed as in danger [1] and requires different conservation methods (*in situ* and/or *ex situ*).

G. petraea and *D. callizonus* are the two rare plant species studied. The distributional range of *G. petraea* (sub-endemic) is Bulgary and Romania [5]. *D. callizonus* is endemic in Romania (Piatra Craiului Massif) [6]. Both are included in the list of endemic plant species recorded within Carpathian alliances [7] and are cited like rare plant species in the red list of superior plant species from Romania [8].

The present paper deals with investigations related with *in vitro* conservation under slow growth conditions at two rare plant species: *G. petraea* and *D. callizonus*.

According to the authors knowledge this is first description into the field of *in vitro* conservation under slow growth conditions without using retardants or other osmolites and also in the field of the evaluation of the effects of slow growth conditions on the genetic stability at biochemical level.

2. Material and Methods

In vitro cultures of the two rare plant species were initiated starting from *in vitro* germinated seeds. The explants (0.5 cm shoots) from the same clone were multiplied by direct organogenesis based on the established protocols [9,10]. The experiment (10 replicates/treatment) was planned to evaluate the responses of the plant species under slow-growth conditions, using seven different media variants (Table 1) and two different temperatures (25°C and 10°C). The media variants used were based on MS formula [11] supplemented with B_5 vitamins and 8g/l Plant Agar (Duchefa) without

growth factors, retardant factors or other osmolites.

Table 1. Media variants used for slow-growth conservation of the two rare plants.

Media variant	Macro elements	Micro elements	Sucrose %
V1	MS	MS	0
V2	MS	MS	0.5
V3	MS	MS	1
V4 (control)	MS	MS	2
V5	MS½	MS½	2
V6	MS¼	MS¼	2
V7	MS1/10	MS1/10	2

The methods tested in this experiment were represented by reducing the carbon source (V1-V3 media variants), reducing the mineral concentration (V5-V7 media variants) [12] (Table 1) and two different temperatures for storage 25º and 10ºC [13,14,15]. The V4 media variant is the control variant represented by 2% carbon source.

In order to evaluate the *in vitro* aspects of medium-term conservation, the health of shoots and rhyzogenesis were registered. The health of shoots is referred to the viable and green explants. The both parameters were calculated as percentage.

The *in vitro* cultures were maintained 12 months with 3 subculture passages for the cultures stored at 25ºC and 2 subculture passages for the cultures stored at 10ºC.

The biochemical analyses were performed after 6 months of *in vitro* culture under different slow growth conditions. The samples used for biochemical analysis are presented in Table 2.

Table 2. The explants used for biochemical analysis maintained in different growth conditions.

Samples	Species	Conditions of *in vitro* cultures
1	*D. callizonus*	V4 media variant at 10°C
2		V7 media variant at 25°C
3		V5 media variant at 25°C
4		V3 media variant at 25°C
5		V4 media variant at 25°C
6		*in vivo* at 25°C
7	*G. petraea*	V4 media variant at 10°C
8		V3 media variant at 25°C
9		V4 media variant at 25°C
10		*in vivo* at 25°C

The pattern of izoenzymes (peroxidases, esterases, glutamate oxaloacetate transferase and malate-dehidrogenase) was studied in the explants prelevated from *in vivo* and from plantlets maintained in different slow growth conditions. The extraction of soluble cytosolic proteins was performed by grinding the plantlets in 0.1 M phosphate buffer, pH 7 at 4^0C. After centrifugation at 15000 rpm for 10 min, the supernatant was used for electrophoretic analysis. Electrophoreses were carried out by the samples migration at 90/120V, 5h, in a discontinuous system using a running gel 8% PAA (polyacrylamide) a stacking gel 4% PAA and a buffer Tris-Gly 0.05M, pH 8.3. After electrophoresis for locating esterase activity, it was used 0.2 % α- and β-naphtyl phosphate as substrate and 0.05% Fast Blue BB in 0.1M phosphate buffer, pH 6.5. The bands were stained in red. For peroxidase activity, 0.08% benzidine in 0.5 M acetate buffer, pH 5 and hydrogen peroxide was used. 10 minutes incubation in 40% methanol and 10% trichloroacetic acid**,** followed by a stained with Coomasie Brilliant Blue for 12 hours were used for proteins. A mixture of PVP, $EDTANa_2$, aspartic acid and cetoglutaric acid was used to view the glutamate oxaloacetate transferase. For malate-dehidrogenase it was used an incubation mix of NBT, PMS and MTT in a Tris buffer added with malic acid, $MgCl_2$ and NAD.

The evaluation of the effects of slow growth conditions on the genetic stability at biochemical level was achieved by observing similarities and dissimilarities between the samples maintained 6 months in different *in vitro* conditions.

3. Results

Reducing carbon source and mineral concentrations

In the case of *G. petraea* species, reducing of carbon source concentration had effects on the morphological and biochemical aspects. The control media variant (V4) allowed the preservation with a good rate of shoots' health (100%) for 12 months but with low rate of rhizogenesis (45%) (Figure 1, 2). The lack of sucrose (V1 media variant) stopped the explants growth. After 60 days of *in vitro* cultures all the explants of both species degenerated on the media with no sucrose.

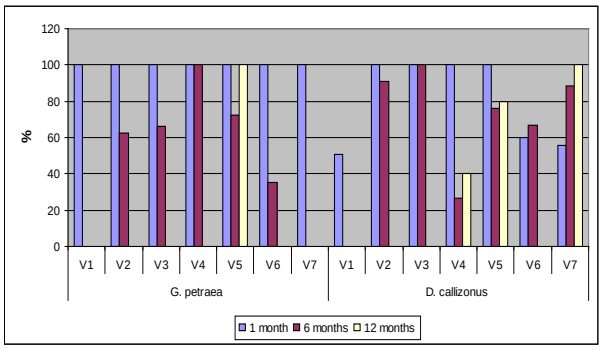

Figure 1. The healthy shoots at *G. petraea* and *D. callizonus* after 12 months of maintained in different slow growth conditions (V1-V7 media variants).

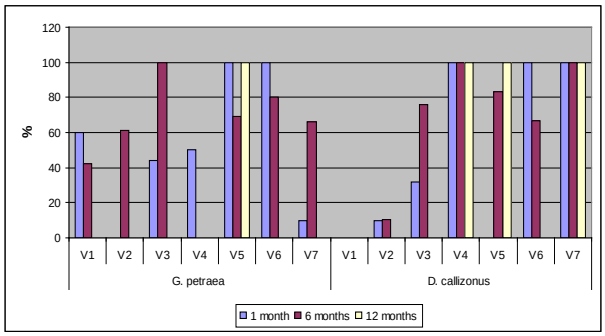

Figure 2. The rhizogenesis at *G. petraea* and *D. callizonus* after 12 months of maintained in different slow growth conditions (V1-V7 media variants).

The hiperhidricity process is present in different percents in all explants maintained of the variants with reduced carbon source (V1 - V3) and mineral concentration (V5 - V7) compared with the control variant (Figure 3).

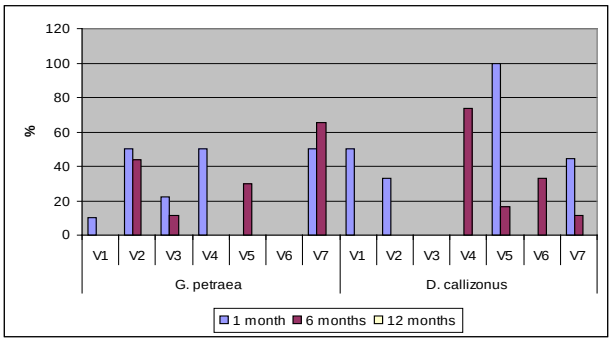

Figure 3. The hiperhidricity at *G. petraea* and *D. callizonus* after 12 months of maintained in different slow growth conditions (V1-V7 media variants).

The V6 media variant (level of mineral concentration reduced at 1/2) allowed the conservation of the species with no hiperhidricity, best shoots viability and rhizogenesis (100%). Having good results concerning the evaluated parameters, the V6 media variant is the only one media variant from the variants tested which allowed the maintenance of medium term cultures for over 1 year (Figure 1).

From the all media variants tested, only the V5 (nutrients reduced at 1/2) and V7 (nutrients reduced at 1/10) media variants allowed the medium term conservation of *D. callizonus* for more than 12 months (Figure 1, 4, 5).

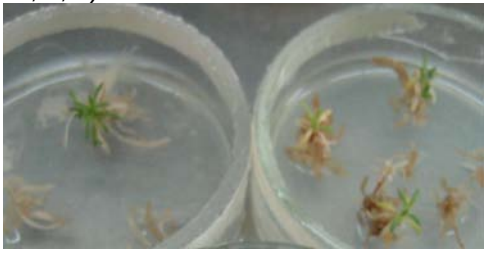

Figure 4. *In vitro* cultures at *G. petraea* under slow growth conditions maintained 12 months on the V5 media variant (MS 1/2).

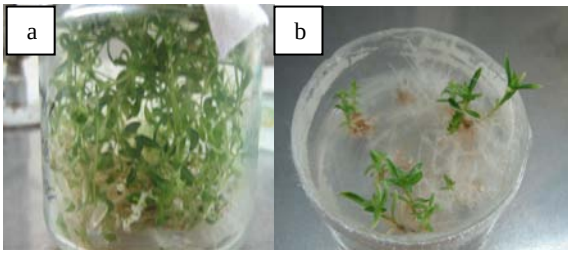

Figure 5. *In vitro* cultures at *D. callizonus* under slow growth conditions maintained 12 months on the V4 (control) (a) and V7 media variant (MS1/10) (b).

Starting with the 6th month, the shoots maintained in these slow growth conditions were characterized by hiperhiricity in different percents (11-73% for *D. callizonus* and 11-65% for *G. petraea*). The rhizogenesis was less developed on the media with reduced carbon source (V1-V3) than on the media with reduced mineral concentration (V5-V7) for both species (Figure 2).

Different temperatures

In order to limit the growth of the two rare plant species analyzed, two different temperatures were used. Between different methods of *in vitro* conservation under slow growth conditions, the change of the storage temperature is the method most used.

The temperature represented by 10°C allowed the maintenance of *G. petraea* more than 12 months in optimal parameters, with a good viability and rhizogenesis rates (Figure 6, 7). The explants maintained for 12 months on the V4 (control) at 25°C were characterized by 100% of hyperhidricity compared with the explants maintained at 10°C (figure 6).

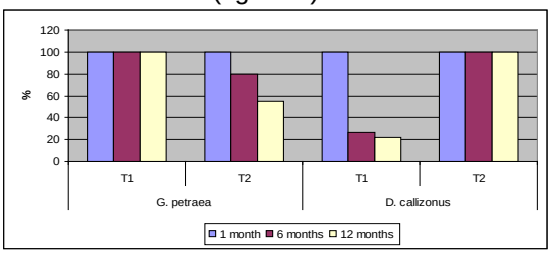

Figure 6. The healthy shoots at *G. petraea* and *D. callizonus* at T1 (10°C) and T2 (25°C) after 12 months of maintained in different slow growth conditions.

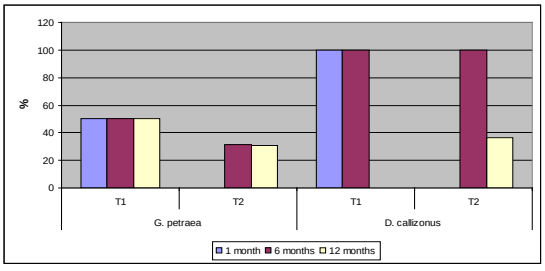

Figure 7. The rhizogenesis at *G. petraea* and *D. callizonus* at T1 (10°C) and T2 (25°C) after 12 months of maintained in different slow growth conditions.

Also, the 10°C temperature was proper for maintaining *D. callizonus* for more than 12 months with a good viability rate (100%) comparing with the explants maintained at 25°C (20%) (Figure 6).

The shoots length of both species was reduced at half comparing with the control temperature (25°C) (Figure 8, 9).

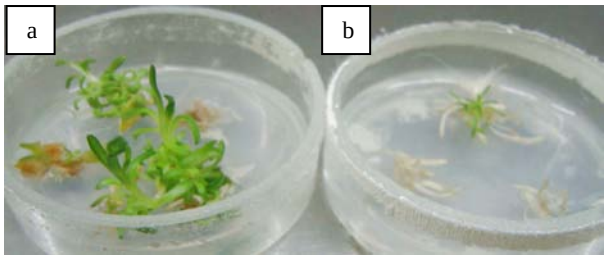

Figure 8. *In vitro* cultures of *G. petraea* maintained 12 months at 25°C (a) and at 10°C (b).

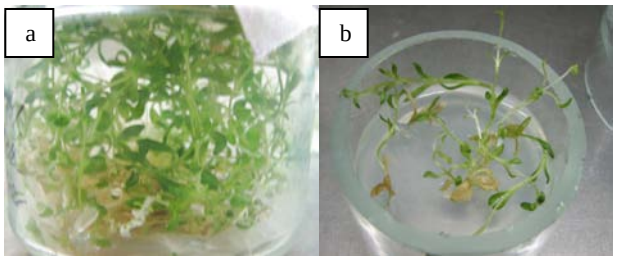

Figure 9. *In vitro* cultures of *D. callizonus* maintained 12 months at 25°C (a) and 10°C (b).

To check the genetic stability of the shoots under slow growth conditions, the biochemical analysis were performed. Although the DNA-based molecular techniques have become increasingly popular in the study of taxonomy and conservation of the endangered plant species, the enzymes spectra are still the basic method for check the genetic stability of the conserved plant species because are able to identify the somaclonal variability arising from the changes induced by *in vitro* culture method.

The enzymatic spectra (1.GOT, 2. peroxidase, 3. esterase, 4. MDH) of the *G. petraea* and *D. callizonus* shoots maintained under slow growth conditions are showed in the figure 10. The physical factors (lack of nutrients and temperature) had some influences on the enzymatic spectra. The biochemical analysis of the samples maintained on the same kind of treatment (reduced sucrose concentration or reduced mineral level) did not shown differences in the enzymatic spectra.

The glutamate oxaloacetate transferase and malate-dehidrogenase patterns relived that there are no significant differences between samples maintained in different slow growth conditions used.

The peroxidase pattern, which is an inducible enzyme, showed that there are differences between the *in vivo* and *in vitro* plants in the case of *G. petraea.* The peroxidase expression depends on the culture condition, physiological, developmental stages [16, 17] and oxidative stress [18, 19].

The pattern of esterases showed minor differences between samples for both plant species analyzed. These differences were observed between samples maintained on the media variants characterized by different physical factors. In the case of *D. callizonus* differences were observed between samples maintained in different conditions like: sample 3 maintained on the media variant V5 characterized by reduced mineral level, sample 4 maintained on the V3 media variant characterized by reduced sucrose concentration and sample 6 *(in vivo* condition).

It is important to underline that no differences were observed between the samples maintained on the same media variant characterized by reduced mineral concentration (samples 2 and 3).

At *G. petraea,* in the pattern of esterases were observed differences between all the samples maintained in the all different conditions (*in vivo*, *in vitro*, low temperature) used.

Concerning the samples maintained at 10°C, minor differences and only in the intensity expression can be observed compared with the sample stored at 25°C (samples 1 and 5 - *D. callizonus* ; samples 7 and 9 - *G. petraea)* (Figure 10).

4. Discussions and Conclusions

In this paper we report the establishment of an efficient medium term preservation protocol based on slow growth conditions for two rare plant species from *Caryophyllaceae* family. It was showed that the slow growth procedures allow clonal plant material to be held in *in vitro* conditions with periodic sub-culturing depending on the species [20]. In this proposed species like *Musa* spp., potato, sweet potato, cassava, yam, *Allium* spp. and temperate tree species are routinely preserved using these kinds of methods [21]. Concerning the storage at low temperature, the first report of successful *in-vitro* storage was of shoot tips of *Vitis rupestris* stored up to 290 days at 9°C [22].

There are some previous data regarding the germplasm conservation of *Caryophyllaceae* ornamentals using cryoconservation [23,24] and using media variant added with different mannitol concentration for some rare *Dianthus* species [25,26,27].

Our results concerning the biochemical analysis of the samples maintained on the same kind of treatment (reduced sucrose concentration or reduced mineral level) did not shown differences in the enzymatic spectra. Similar results were obtained at *in vitro* regenerants of *Marsilea quadrifolia* [28]. Also, in the case of the species *Veronica multifida ssp. capsellicarpa* maintained for 8 months in a medium- term protocol based on media variant supplemented with mannitol showed no differences between regenerants [29].

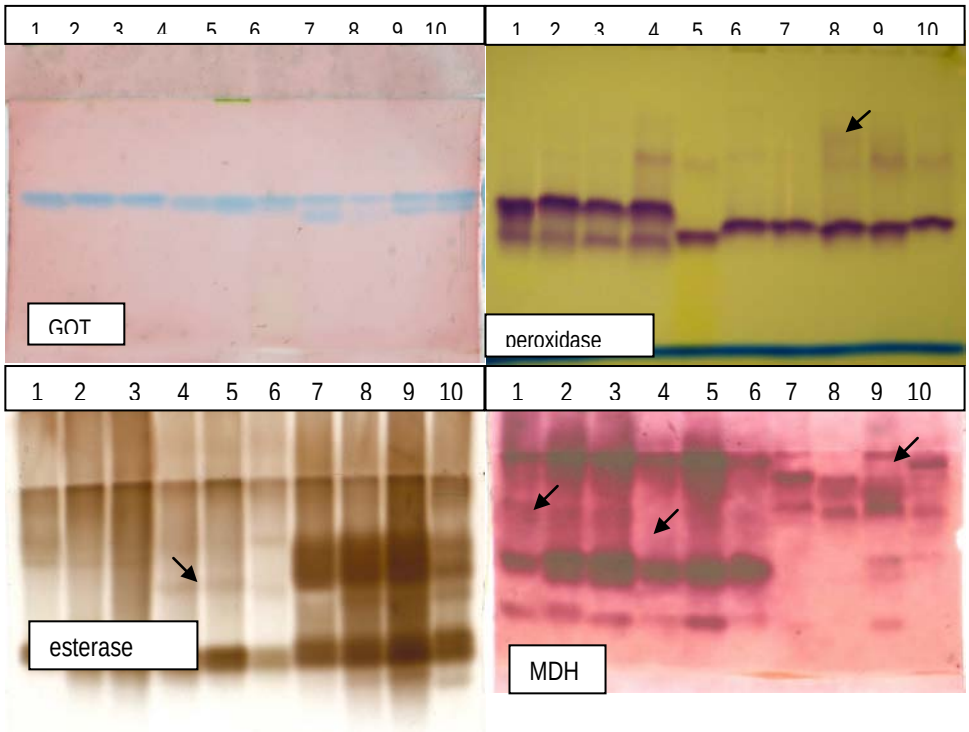

Figure 10. Enzymatic spectra of the shoots maintained under slow growth conditions of *D. -callizonus* (1-6) and *G. petraea* (7-10). 1 -V4 media variant at 10°C, 2 - V7 media variant at 25°C, 3 - V5 media variant at 25°C, 4 - V3 media variant at 25°C, 5 - V4 media variant at 25°C, 6 - *in vivo* at 25°C, 7 - V4 media variant at 10°C, 8 - V3 media variant at 25°C, 9 - V4 media variant at 25°C, 10 - *in vivo* at 25°C. The arrows represent the presence or the absence of the bands between the samples maintained in different slow growth conditions

Our data concerning the *in vitro* conservation of rare plant species using slow growth conditions are in accordance with those reported by Botău et al., 2005. In this work a cheap protocol of slowing plants growth that uses *in vitro* cultures and low temperatures (16°C and 24°C) was established [30]. Also, slow growth conditions (reduced mineral and sucrose concentrations) were used to preserve 14 species of *Dioscorea* spp. more than 2 years with passages at 6-8 months [31]. Low temperature storage of *in vitro* cultures appears to be a highly promising method for storage of *Rauvolfia serpentina* Benth. ex Kurz [32] which was stored 15 months at 15°C.

An efficient medium term preservation protocol based on reduced mineral concentration at 1/4 for *Gypsophilla petraea* (Baumg.) Reichenb and 1/10 for *Dianthus callizonus* Schott&Kotschy at 25°C was established. We showed that the storage at 10°C is a good way to maintain the analyzed species for more than 12 months with a reduced number of subcultures (2). Because no significant differences were observed between the samples maintained in slow growth conditions, these two rare plant species may be preserved by *in vitro* culture medium term without genetically changes at biochemical level.

Acknowledgements

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