IN VITRO SEED GERMINATION IN THREE RARE TAXA FROM THE ROMANIAN CARPATHIANS FLORA

CATANĂ Rodica, HOLOBIUC Irina, MOLDOVEANU Mirela

Abstract. Germination of the seeds is the primary step for plant development *in vivo* and also *in vitro* conditions. The seed germination rates vary between different species, altitudes of the habitats, site locations and years, but without clear trends. In the case of endangered plant species the number of seeds may be limited or they have problems concerning their germinability. In this context, an evaluation of the seed germination under *in vitro* (using different temperatures, media variants and pH values) and *ex vitro* conditions in three rare plant species belonging to different families was made. This aspect is important for conservative purpose. Rare plant species are considered of interest because of their ability to adapt to extreme or limitative environmental conditions. The studied species were *Erigeron nanus*, *Dianthus callizonus, Papaver alpinum* ssp. *corona-sancti-stefani.* In *ex vitro* conditions, only *E. nanus* and *D. callizonus* germinated. In *P. corona-sancti-stefani*, seed germination was induced only in *in vitro* conditions with low germination rate (10%) at 4°C. In the case of *E. nanus,* only media variant added with sucrose (30g/) allowed a good germination rate (66.66%). The seeds of *D. callizonus* germinated better on the media variant with sucrose (30g/l) and higher pH value (6.8).

Keywords: rare plants, seed germination, *in vitro, ex vitro* culture.

Rezumat. Germinarea in vitro a seminţelor la trei specii de plante ameninţate din flora Carpaţilor din România. Germinarea seminţelor reprezintă prima etapa în dezvoltarea plantelor atât *in vivo* cât şi *in vitro*. Rata de germinare a seminţelor variază între specii, în funcţie de altitudine, de locul şi anul recoltării. Întrucât la speciile de plante rare pot apare probleme privind viabilitatea şi germinarea seminţelor, a fost realizată evaluarea germinării seminţelor în condiţii *in vitro* (diferite temperaturi, variante de medii de cultură şi pH) şi *ex vitro,* la trei specii de plante din familii diferite. Acest aspect prezintă importanţă conservativă. Aceste specii de plante ameninţate sunt considerate de interes datorită capacităţii lor de a se adapta la condiţii de mediu extreme sau limitante. Speciile luate în studiu au fost *E. nanus*, *D. callizonus, P. alpinum* ssp. *corona-sancti-stefani*. În condiţii *ex vitro,* au germinat numai seminţele de *E. nanus* şi *D. callizonus*. La specia *P. corona-sancti-stefani* a fost indusă germinarea seminţelor menţinute la 4°C, în condiţii aseptice însă cu rate mici de germinare (10%). În cazul seminţelor de *E. nanus* o rată bună de germinare (66,66%) a semintelor în condiţii *in vitro* a fost obţinută pe mediu adiţionat cu 30g/l şi pH 5,8, fără factori de creştere. Seminţele de *D. callizonus* au germinat mai bine pe varianta de mediu adiţionată cu 30g/l, fără factori de creştere şi pH 6,8.

Cuvinte cheie: plante rare, germinarea seminţelor, condiţii *in vitro, ex vitro.*

INTRODUCTION

The seed represents the main way of plant reproduction and is one of the key factors that determine the species maintenance in time and space (VENABLE & LAWLOR, 1980). Its storage may cause the loss of its germinative capacity (AMORIM et al., 1997).

In the case of threatened, rare and endemic plant species, knowing the seed germination requirements is useful for their conservation and management. Seed germination is important for the preservation, especially for **ex situ** strategies and reproduction of rare and endangered plant species (PORTEOUS, 1993; CLEMENTE & HERNÁNDEZ, 1995; BURMEIER & JENSEN, 2008; KAGAYA et al., 2008). It is recommended to use seeds for propagation of the rare and endangered plant species (VAN WYK & SMITH, 1996). The success of rare plant conservation programs depends on the knowledge of the seed germination behaviour, being considered important in developing effective protocols for promoting **ex situ** conservation (FENNER & THOMPSON, 2005). Seed germination is also used as initial explant for obtaining *in vitro* tissue culture for secondary metabolites (VERPOORTE, 2000).

The rare plant species are that species whose taxa have small populations worldwide, which are currently not threatened but are at risk (due to their restricted area) (OLTEAN et al., 1994). Because the rare taxa are exposed to possible extinction (MACE & LANDE, 1991), studies carried on them are considered important both of practical point of view concerning biodiversity conservation (MYERS et al., 2000) and also of theoretical value due to their ability to adapt to extreme conditions.

In the case of rare plant species, some problems concerning seed viability and germination can occur.

The **ex situ** conservation means the preservation of plant species out of their natural habitats and it is ensured by **ex situ** collections (seed banks, gene banks, botanical gardens and the use of *in vitro* techniques) (IPGRI / FAO, 1996). The *in vitro* techniques can be used to optimize the culture conditions to enhance the seed germination.

Papaver alpinum L. ssp. *corona-sancti-stefani* (ZAPAL.) BORZA (Papaveraceae family) (Fig. 1) is a rare (OLTEAN et al., 1994; OPREA, 2005; CIOCÂRLAN, 2009) and endemic plant species for South Eastern Carpathian Mountains (CIOCÂRLAN, 2009). The species has a scientific importance being the only representative of the genus in the alpine Flora. Also, the species plays an important role as pioneer plant fixing the detritus on the rocky substrate. The limitative factors for this species are tourism and detritus crumbling (DIHORU & PÂRVU, 1987).

Erigeron nanus SCHUR (Asteraceae family) (Fig. 1) is considered as vulnerable /rare species (OLTEAN et al.,1994; OPREA, 2005) and endemic plant species (CIOCÂRLAN, 2009; WITKOWSKI et al., 2003). Also, it was considered as a species of European concern (OZINGA et al., 2005).

Dianthus callizonus SCHOTT & KOTSCHY (Caryophylaceae family) (Fig. 1) is an endemic rare alpine flower native in a small area of South Eastern Carpathians Mountains. It is valuable from a scientific raison because of its genetics and taxonomy and because of the possibility to form hybrids with *D. spiculifolius* and *D. tenuifolius* (DIHORU & PĂRVU, 1987).

Figure 1. *P. corona sancti stefani* (photo: Catană Rodica), *E. nanus* (source net), *D. callizonus* (photo: Holobiuc Irina) in natural habitats.

Our aim was to evaluate the seed germination in different conditions in the case of three rare plant species belonging to different families in order to improve the seed germination.

MATERIAL AND METHODS

The plant material was represented by mature seeds. The plant material was collected in 2006 and 2008 from the natural habitats. The germination rate was tested in 2009 for all three species.

In the case of *E. nanus* the seeds were collected from Piatra Craiului Massif in 2006.

The evaluation of the seed germination was realized on the media variants added with different growth factors (Table 1), in two different temperatures and light/dark regime.

In the case of *P. corona-sancti-stefani,* part of the seeds was obtained from the Botanical Garden Cluj-Napoca "Al. Borza" in 2003 and also some of them were collected from Piatra Craiului Massif in 2008.

In the case of *D. callizonus*, *in vitro* germination of the seeds collected from two sites, in two years and on media variants with different pH values.

The TTC test was made in accordance with ISTA international standards. The TTC is used to differentiate metabolic active and inactive tissues and to check the seeds viability. 1% tetrazolium chloride (2,3,5-triphenyl-2H-tetrazolium chloride) was prepared in distilled water with pH 6.5. The seeds were immersed in TTC solution and kept at room temperature in the dark for 24 hours.

***In vitro* seed sterilization.** The seeds were first washed for 2 hours in running tap water, followed by $HgCl_2$ 0.1 % treatment for 10 minutes and finally, three washing in sterile distilled water were done.

The **germination rate** (%) represents the number of germinated seeds/ the number of total seeds x100. The germination rate was tested **in vitro** (at different temperatures, media variants, pH value) and *ex vitro* conditions.

For *in vitro* germination, the sterilized seeds were inoculated on the Murashige and Skoog basal media variant (MURASHIGE & SKOOG, 1962) without plant growth factors at a 16/8 photoperiod 4ºC and 25ºC for *P. corona-sancti-stefani.*

In *E. nanus,* four media variants (Table 1) were tested at 4ºC and 25ºC.

Table 1. Media variants tested for *E. nanus* seed germination.

Media variants	Basal media	Sucrose (g/l	Growth factors (mg/L)
S1	MS	30	BAP 1, NAA 0.1
S2	MS	30	-
S3	MS	-	-
S4	MS	30	GA_3 100

Legend: MS - Murashige & Skoog medium, (MURASHIGE & SKOOG, 1962); BAP - benzylaminopurine; NAA - alfa-naphtyl acetic acid; GA_3 - gibberellic acid.

In the case of *D. callizonus,* MS without growth factors and three pH values was used.

For *ex vitro* germination, the seeds were placed in Petri dishes on the filter paper in distilled water. The Petri were kept in 25ºC and a 16/8 photoperiod.

A number of 60 seeds per plant species was tested for the evaluation of the seed germination using three repetitions / treatment variant.

A seed was considered to be germinated when its radicle was emerged.

Statistics. The results were assessed using the 1-way ANOVA created by Daniel's XL Toolbox version 5.04. A Bonferroni-Holm posthoc test was used.

RESULTS AND DISCUSSIONS

Knowing the seed germination rate is relevant for plant ecology. In the case of alpine plant species, the asynchronous germination rate may be considered as an adaptation to the environment, where medium controlled germination can be crucial to seedling survival (PELTON, 1956; AMEN, 1966; BLISS, 1971).

Previous studies concerning seed germination of the threatened plant species were done, but a specific methodology for germination of seeds belonging to the alpine taxa was not found (KORNER, 1999). The germination behaviour can vary within a single species from one population to another, from year to year and among individuals (URBANSKA & SCHUTZ, 1986).

A lot of studies concerning the evaluation of seed germination of rare plant species in non-sterile and sterile conditions were performed (CUEVAS & FIGUEROA, 2007; KANDARI et al., 2008; KADIS et al., 2010; SAYANIKA DEVI et al., 2012; ABDOLLAHI et al., 2012).

The storage conditions of the seeds are one of the many other factors which can influence the seed quality expressed as seed viability and vigour. It is known that there are differences between species concerning seed longevity (how long seeds can be stored under given conditions). This aspect is crucial for an effective management of seed conservation collections (PROBERT et al., 2009).

The TTC test is an important rapid seed viability test based on the activity of dehydrogenases that catalyse mitochondrial respiration. In the living tissues of the seeds, dehydrogenases convert the TTC to formazan (NETO et al., 1999) an insoluble red compound that stains in red the living tissues. The presence of formazan allows the differentiation between living and inactive metabolic tissues (FILHO, 1999).

The TTC test results in the case of the three threatened plant taxa are shown in Table 2.

Table 2. TTC test performed on the seeds of three different plant taxa.

Species	Collected data	Sources	TTC reaction
P. corona-sancti-stefani	2003, 2008	Botanical Garden Cluj, Piatra Craiului	– +++
E. nanus	2006	Piatra Craiului	++
D. callizonus	2004, 2006	Piatra Craiului	+ +++

Legend: +++ good reaction, ++ low reaction, + very low reaction, - no reaction.

Germination of the seeds is a complex physiological process determined by the imbibitions of the tissues with water, after the dormancy mechanisms were delayed. The seed germination depends on the internal (endogenous plant hormones) and external conditions (temperature, water level, oxygen content and light or darkness regime) (RAVEN et al., 2005; SEN, 2010). Also, it is already known that there are differences concerning the germination requirements connected to the geographical distribution (PROBERT, 2000).

In our case, the seed germination in sterile conditions (*in vitro*) was higher than in non-sterile conditions (*ex vitro*) for the all three plant taxa (Table 3).

In *ex vitro* conditions, only *E. nanus* and *D. callizonus* have germinated.

In the case of *in vitro* conditions, the seed germination was induced at low rate in *P. corona-sancti-stefani* and improved in *E. nanus* and *D. callizonus*.

Table 3. Formation of seedlings and % of seed germination in the three threatened taxa.

Species	Conditions	No. of seeds	Mean no. of seedlings	% of seed germination	P value	F Critical
P. corona-sancti-stefani	*In vitro*	30	4	13.33*	0.01613	16
	Ex vitro	30	0	0		
E. nanus	*In vitro*	30	21	70*	0.03759	9.375
	Ex vitro	30	6	20		
D. callizonus	*In vitro*	30	24	80*	0.047421	8
	Ex vitro	30	14	46.66		

Legend: Values marked by * are significantly different between *in vitro* and *ex vitro* conditions at $p < 0.05$ using the posthoc test Bonferroni-Holm.

Some *in vitro* studies were done concerning regeneration in *Papaver* species starting from seeds (TISSERAT & BERHOW, 2009; ZAKARIE et al., 2011). Also, a comparative study of seed germination ecology in four *Papaver* taxa was made (KARLSSSON & MILBERG, 2007).

In the case of *P. corona-sancti-stefani*, the seeds collected in 2003 showed no TTC positive reaction and no germination rate neither in *ex vitro* nor in sterile *(in vitro)* conditions (Table 2). The seeds collected in 2008 germinated only in sterile conditions. There are some studies concerning the germination requirements which differ from year to year (KAYE et al., 1999). The variation in germination characteristics could be interpreted as one of the most important survival strategies for species growing under unpredictable environmental conditions (GUTTERMAN, 1994; KIGEL, 1995).

Two different temperatures (25°C and 4°C) were tested for the seeds collected in 2008 to induce the germination in vitro. After 45 days, the germination rate of the seeds was low, with non-significant differences registered between the tested temperatures (Fig. 2).

Despite of the data which proved that the seeds belonging to *Papaver* genus germinate and growth at 20-25°C (TISSERAT & BERHOW, 2009; GORGOROV et al., 2011), in our case, *P. corona-sancti-stefani* seeds germinated better at 4°C.

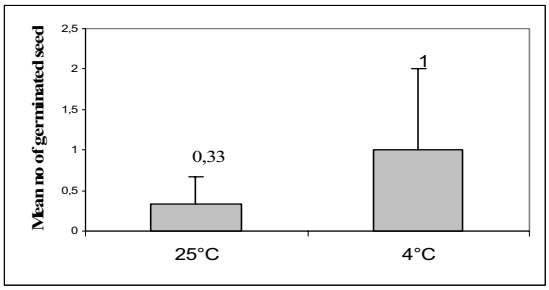

Figure 2. Mean number of *P. corona-sancti-stefani* seeds germinated at 25°C and 4°C.

Some studies in *Erigeron* species concerning seed germination in laboratory conditions were made (CUEVAS & FIGUEROA, 2007).

In the case of *E. nanus* SCHUR**,** four media variants, two different temperatures and light/dark regime were tested to increase the germination rate. A high percentage of seed germination of *E. nanus* (66.66%) was obtained at 25°C in the presence of light, while at 4°C in the dark, the seed germination rate was inhibited (Fig. 3). According to the literature, more than 86% of the species require light to germinate without requiring cold treatment. The data obtained are in concordance with studies on seed germination ability in other species of rare plants (KOORNNEEF et al., 2002).

The seed germination is genetically determined and affected by endogenous plant hormones (BENTSINK & KOORNNEEF, 2002). The applications of exogenous plant growth factors (PGR) have been extensively used for enhancing the seed germination and the development of seedlings in the laboratory. There are several studies regarding the influence of cytokines on the germination capacity of seeds in different species (NIKAM & BARMUKH, 2009; NIKOLIĆ et al., 2006). The gibberellins are most prominent growth regulator used (SHUE-LOCK, 1968; CERABOLINI et al., 2004).

In our case, the addition of BAP (1mg/l) in one medium variant had no significant effect on the seed germination, this being lower than the rate obtained in the case of S2 medium variant (MS medium without PGR).

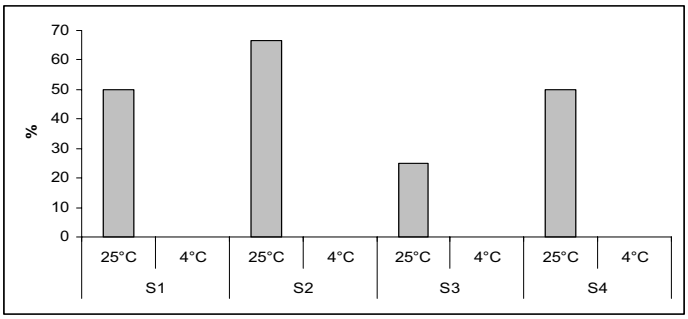

Figure 3. The seed germination rate of *E. nanus* at 25°C and 4°C and light/dark regime.

S4 variant added with 100mg/l GA_3 allowed about 20% of the seeds to germinate (Fig. 4). Between the four media variants tested for seed germination there are statistically significant differences ($p < 0.05$).

The four tested media variants (S1-S4) (Table 2) allowed the seeds to germinate with different rates, with good results on the S2 media variant added with 30g/l sucrose (Fig. 4).

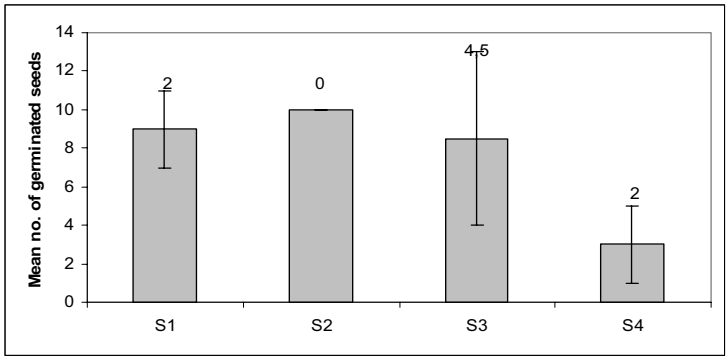

Figure 4. The mean number of *E. nanus* seeds germinated on S1-S4 media variants.

There are some studies concerning the seed germination in Caryophyllaceae family. A comparative research concerning germination of the seeds collected from different side of Europe was done (THOMPSON, 1970). Other studies of the seed size variation in the populations in relation to habitat conditions were performed in *Silene dioica* (L.) CLAIRV (THOMPSON, 1981). Studies concerning *in vitro* seed germination in *Dianthus* species were made in *D. nardiformis* JANKA (HOLOBIUC et al., 2009), in *D. ciliatus* ssp. *dalmaticus* and *D. giganteus* ssp. *croaticus* (RADOJEVIĆ et al., 2010), in *D. henteri* (CRISTEA et al., 2010), in *D. barbathus* (LĂPĂDĂTESCU et al., 2012).

In our case, the *D. callizonus* seeds tested in *ex vitro* conditions showed over 40% germination rate. These data are in accordance to those obtained by MICLE in 1967, who reported that the germination of *D. callizonus* seeds in natural habitat was around 35-50% (MICLE, 1967).

Comparing the germination rate of the seeds collected in 2004 and 2006, the seeds collected in 2004 lost their germinability (Fig. 5). These data are in accordance with the data of KAYE et al., 1999.

The seeds collected in 2006, are originated from two different sites of Piatra Craiului Massif: Piatra Craiului Mică (1811 m altitude) and Padina Popii (2018 m altitude). The distance between the two sites is ~ 20 km. In our case, non-significant differences were obtained concerning the seed germination rate from the two sites (Fig. 4). The same results were reported by GIMÉNEZ-BENAVIDES et al., 2005 in 20 endemics species of the Iberian Peninsula who did not find a consistent pattern in the germination rate related to altitude variation.

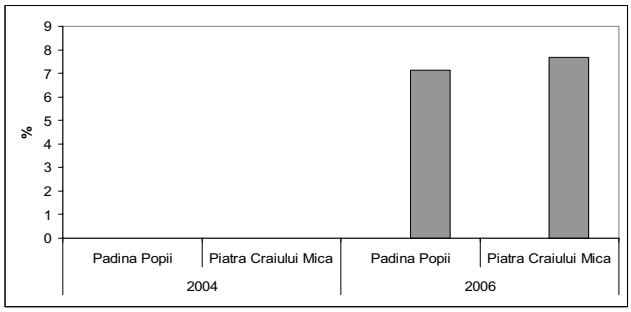

Figure 5. The germination rate of *D. callizonus* seeds collected in 2004 and 2006 from two different sites.

Other important factor that can influence the seed germination is the pH value. Three different pH values (4.8; 5.8 and 6.8) were tested. The higher number of germinated seeds was obtained on the medium variant with 6.8 pH (Fig. 6). The differences between the three used pH values were statistically non-significant ($p < 0.005$). These results are in accordance with the data recorded in the natural habitats, *D. callizonus* being an alpine species growing on calcareous substrate with 6-7.5 pH (ONETE, 2011). Our data in *D. callizonus* are similarly to the experiment made in S*alvia* sp. where the 5.0 to 6.5 pH values increased seed germination (SHOEMAKER & CARLSON, 1990).

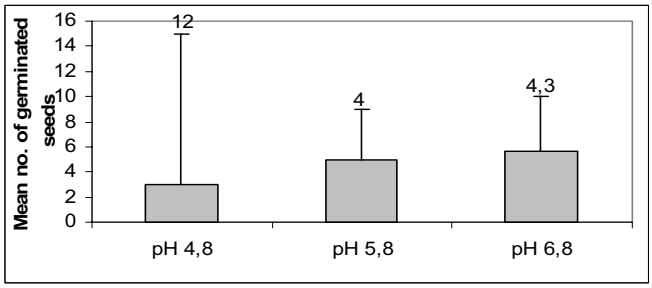

Figure 6. The mean number of germinated seed of *D. callizonus* at different pH values.

The plants obtained through *in vitro* seed germination were used for the establishment of short-term preservation protocol in *E. nanus* and *D. callizonus* (HOLOBIUC et al., 2005; HOLOBIUC & BLÎNDU, 2006; BLÎNDU & HOLOBIUC, 2007), for *in vitro* conservation under slow-growth conditions during medium-term (HOLOBIUC & BLÎNDU, 2006; CATANĂ et al., 2010a). In *E. nanus, in vitro* regenerants obtained from one germinated seed was used to assay the genetic stability (CATANĂ et al., 2010b).

CONCLUSIONS

The seed germination rates were improved in the case of *in vitro* conditions in all three taxa tested.

Seeds of *P. corona-sancti- stefani* germinated better at 4°C.

Seeds of *E. nanus* germinated at 25°C in the presence of light on the medium variant added with 30g/l sucrose, without plant growth factors and 5.8 pH value.

For *D. callizonus* seeds germination, it is suitable a medium added with 30g/l sucrose without plant growth factors, at 6.8 pH.

In vitro germinated seedlings were used for further studies concerning *in vitro* preservation during short and medium-term preservation.

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