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**FLAGSHIP SPECIES OF THE PIENINY NP. PROTECTED *EX SITU* AT PAS BG CBDC SEED BANK
AT WARSAW-POWSIN**

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ABSTRACT: Pieniny Mts. are important biodiversity hotspot in Central Europe. According to Pieniny National Park Long Term Conservation Plan 2000-2020 small, isolated populations of some of Pieniny NP flagship species can become extinct till 2020. That is why their *ex situ* preservation at seed bank is justified and reasonable as supplement of active and passive *in situ* conservation measures carried out by National Park staff. PAS Botanical Garden – Center for Biological Diversity Conservation at Warsaw-Powsin has a long tradition of cooperation with Pieniny National Park (S Poland). This article focuses on the actions undertaken during EU funded conservation project: “*Ex situ* conservation of native, threatened and protected plant species in eastern Poland - FlorNaturOB”. Seeds of *Dendranthema zawadzkii*, *Erysimum pieninicum* and *Minuartia setacea* collected by „FlorNaturOB” team at Pieniny NP: (1) showed orthodox behaviour (could be easily dried and frozen at ultra-low temperatures of liquid Nitrogen), (2) do not exhibit seed dormancy. Species’ localization and collecting time influenced the seed samples quality.

KEY WORDS: Pieniny NP special interest plant species, *Dendranthema zawadzkii*, *Erysimum pieninicum*, *Minuartia setacea*, cryopreservation, seed dormancy, “FlorNaturOB” project

Introduction

Carpathians are northernmost centre of plant endemism in Europe. Polish-Slovakian Pieniny Mts. due to their limestone rocks, unique orography (steep-sided cliffs and gorges), climatic conditions and geological history are important biodiversity hotspot of Central Europe (Pawłowski 1970; Zarzycki 1976; Smólski 1982; Benčat'ová 2001; Mirek and Piękoś-Mirkowa 2009). During Pleistocene the Pieniny mountain range had been a refugium for xerothermophilous flora and fauna, including Ponto-Panonian, Irano-Turanian and Submediterranean elements. During Holocene this small mountain range became migration route for:

- alpine plant species descending from Tatra Mts. at Małopolska and Roztocze Uplands e.g.: *Dryas octopetala* L., *Aster alpinus* L.;
- steppe and thermophilous oakwood species recolonizing Central and Northern Europe e.g.: *Adenophora liliifolia* Bess., *Crepis praemorsa* Tausch, *Inula ensifolia* L., *Senecio integrifolius* (L.) Clairv. etc. (Bodziarczyk 2008; Kaźmierczakowa 2008; Piękoś-Mirkowa 2008; Zarzycki and Wróbel 2012).

Alkaline soils and range isolation are key factor enhancing speciation (Favarger 1972; Hendrych 1982; Tribsch and Schönswetter 2003; Piękoś-Mirkowa and Mirek 2009). That is why Pieniny flora has many neoendemic varieties and small species, with recognizable but disputable taxonomic rank e.g.: *Erysimum pieninicum* (Zapał.) Pawł., *Minuartia setacea* (Thuill.) Hayek var. *pienina* (Zap.) Pawł., *Centaurea triumfetti* All. var. *pieninica* Pawł. (Pawłowska 1953; Pawłowski 1970; Zarzycki 1976; Piękoś-Mirkowa and Mirek 2003, 2009; Fig. 1,2). The oldest Pieniny endemic is *Taraxacum pieninicum* Pawł., which evolved in Paleogene (Pawłowska 1953; Małecka 1962; Prochazka et al. 1999; Wróbel 2004, 2008). Extinction risk assessment of the flora of the Polish Pieniny was published by Szczocarz et al. (1992), Wróbel (2003), Wróbel and Zarzycki (2010), and Zarzycki and Wróbel (2012).

Wróbel (2003) claims that „*during the enforcement of National Park Conservation Plan some minute, isolated populations of flowering plant species such as: Androsace lactea, Taraxacum pieninicum, Lilium bulbiferum, as well as some orchid species and species associated with wet and humid habitats, can become extinct at Pieniny range.*”

Ex situ preservation of germplasm of the above mentioned seed bank seems reasonable and justified as supplement of active and passive *in situ* conservation measures carried out by National Park staff. PAS Botanical Garden – Centre for Biological Diversity Conservation at Warsaw-Powsin has a long tradition of cooperation with Pieniny National Park (S Poland)

(Galera et al. 2000; Puchalski 2000; Zarzycki and Lankosz-Mróz 2000; Gasek et al. 2004; Puchalski and Gawryś 2007; Puchalski et al. 2011, 2013; Trejgell et al. 2013).

Material and methods

Conservation measures had been carried out in the frameworks of EU program: „*Ex situ* conservation of native, protected and threatened species in Eastern Poland. Polish: Ochrona *ex-situ* dziko rosnących, zagrożonych i chronionych roślin w Polsce wschodniej – FlorNaturOB” in the years: 2010 – 2013”.

Seeds were collected and prepared for long-term preservation at Seed Bank according to ENSCONET standards, described in international manuals (<http://ensconet.maich.gr/Download.htm>). Seeds viability has been tested using PAS BG CBDC Seed Bank own methods (Muranyi 2002 unpubl.; Muranyi and Wróbel 2006; Puchalski et al. 2013).

Seeds biology of three Pieniny National Park flagship (charismatic) species: *Erysimum pieninicum* (Zapał.) Pawł., *Minuartia setacea* (Thuill.) Hayek var. *pienina* (Zap.) Pawł. and *Dendranthema zawadskii* (Herb.) Tzvelev, had been studied in detail (tab.1).

After gathering and delivering to bank seeds of above mentioned species were cleaned (using mechanical seed separator) and dried to 20% moisture content (15°C, 20% RH). 30 days later, when seeds moisture reached 6-8% its viability, germination biology (germination tests in environmental chamber with controlled conditions of temperature, light and humidity) has been checked. Due to previously observed lack of dormancy their viability has been tested through germination on paper moistened with distilled water, at climatic chamber, applying conditions adopted in the past by Muranyi for Pieniny rare species germination: 25°C/15°C (16h/8h), 70% RH (Muranyi 2002 unpubl.). To avoid pathogenic microfungi seeds had been treated with Funaben T fungicide during the germination tests. Due to small number of gathered seeds only 25 seeds of each Pieniny flagship species were used to each germination test and the tests had been repeated twice a week.

After checking their germination ability, the second experiment were test for freezing tolerance in LN2. It has been applied by: direct immersion in LN2 or slow freezing (0,5°C/minute) according to control, non-frozen sample. After 30 days of storage in vapour of LN2 (ca. -160°C) the seed germinability was again tested. In case of no differences between frozen and control samples the particular seed sample was transferred for long-term storage in cryogenic vaults. *E. pieninicum* and *M. setacea* seeds were examined in two repeats of 25 seeds each, *D. zawadskii* generative diaspores has been examined in three repeats also 25

seeds each. Each freezing tolerance test for *E. pieninicium* and *D. zawadskii* seeds had three options:

- direct immersion of seeds at LN₂ vapours (circa -160°C)
- gradually cooling of seeds at IceCube device (0,5°C/min.) to LN₂ temperature (circa -160°C)
- unfrozen control (K).

In case of *M. setacea* seeds we had to resign from gradually cooling of seeds at IceCube device due to small number of available seed material.

After 30 days in cryogenic vault the germinability of frozen seeds has been examined again using previously described, standard Powsin Seed Bank methods. Above mentioned viability and freezing tolerance tests were necessary due to ensure that material gathered will remain viable after cryostorage.

Results

Three Pieniny NP. flagship species collected at four natural sites has been secured *ex situ* at PAS BG-CBDC Seed Bank (Tab. 1). Carried out standard viability and freezing tolerance tests confirmed *D. zawadskii*, *E. pieninicium* and *M. setacea* seeds excellent capacity to long-term cryostorage.

Above mentioned seed material had fully withstand drying to 6-8% RH. Seeds do not exhibit dormancy and its germinability were significant (Tab. 2). Only *E. pieninicium* seed sample gathered at 2010 had not germinated successfully and achieved only 8% of viability in our, standard germination test. We carried out additional tests using GA₃ (400 ppm) to coerce germination. After supplementation with gibberelic acid seeds from this sample germinated in 76%, but obtained seedlings were weak and they were dying out fast. Next we differentiated time of seeds exposition to GA₃ from one to seven days. In each options seeds germinated but the seedlings died soon after germination.

That is the reason why we decide to repeat the gathering of *E. pieninicium* seed material at Pieniny NP. Material collected at 2011 had been checked and proved itself to be viable, germinating up to 86%. The reason of negligible germinability of 2010 seed lot were probably too early time of collecting – July instead of August-early September. Although *Erysimum*' siliquae seemed mature at July 2010 there were too young for gathering to seed bank (Table 2).

In case of *D. zawadzkii* seed material germinability were very high (94% from Okrąglica, 76% from Wąwóz Sobczański population). *M. setacea* sample collected at 2010 germinated in 80%.

Carried out freezing tests proved that all Pieniny flagship species remain viable after long-term cryostorage (Tab. 2). In most cases no significant differences between control and deeply frozen samples has been observed. *D. zawadzkii* seed material from Wąwóz Sobczański germinability of the directly immersed at LN2 sample were even higher (68%) than control (52%). Only *M. setacea*' seeds germinability slightly decreased after freezing.

After standard tests all gathered Pieniny flagship seed samples (excluding *E. pieninicum* from 2010) have been safeguarded in Powsin Seed Bank deposit. Duplicates of above mentioned samples had been secured at Forest Gene Bank "Kostrzyca" near Karpacz to enforce total safety of the germplasm (Puchalski et al. 2013).

Discussion

According to Piękoś-Mirkowa and Mirek (2010) „one of most important, current tasks of botanic gardens should be ex situ conservation of Polish endemics, especially endemics in the strict sense of the word. Modern botanic gardens' activity remains insufficient to conservation needs”.

European funded program „*Ex situ* conservation of native, protected and threatened species in Eastern Poland – FlorNaturOB. Polish: Ochrona *ex situ* dziko rosnących, zagrożonych i chronionych roślin w Polsce wschodniej - FlorNaturOB” could has been a remedy to this negligence.

Effective, long-term preservation of Pieniny endemics and relict's germplasm by seed cryopreservation require a good knowledge on their germination biology and optimization of seeds drying, germinating, freezing and refreezing. According to Ellis and his collaborators (1985) drying is the most important procedure in seeds safeguarding. Fortunately all three Pieniny flagship species produce *orthodox* seeds. They can be easily dry to very low humidity and secured at liquid nitrogen. Only *Minuartia setacea*' generative diaspores revealed some loss of germinability after cryostorage. However unpublished Muranyi observations from 2002 proved that even *Minuartia setacea*' seeds remained viable in 80% after cryostorage. Obtained results also confirm that testing of each seed sample germinability is a necessary procedure (Table 2). Sample quality can be influenced by gathering locality (*D. zawadzkii*), date of collecting (*E. pieninicum*) as well as other factors contribute to total sample quality, and reaction on liquid Nitrogen (Tab. 2).

Even the best *in situ* protection at National Parks and nature reserves can be insufficient due to: abiotic factors (avalanches, wildfires, floods, droughts, windthrows), pathogen and herbivore activity, illegal plant gathering, genetic drift and shift etc. This is the reason why *in situ* protection ought to be supported by *ex situ* conservation. Seed cryopreservation proved to be useful tool in long-term securing of the Pieniny flagships conservation outside their natural habitats.

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**INFRASTRUKTURA
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NARODOWA STRATEGIA SPÓŁNOŚCI



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Figure 2. Flowering *Erysimum pieninicum* at Czorsztyn Castle Hill, Pieniny NP (phot. A. Nowak).



Figure 3. *Minuartia setacea* at Okrąglica, Pieniny NP (phot. A. Nowak).

Tab. 1. Species protected in Seed Bank of PAS BG CBDC in 2010-2013 period.

Species name*	Category	Localization	Other information
		of threat**	
<i>Minuartia setacea</i> (Thuill.) Hayek	CR	Okrąglica	Steppe relic
<i>Erysimum pieninicum</i> (Zapał.) Pawł.	VU	Góra Zamkowa at Czorsztyn	Polish neoendemic (stenochoric) species
<i>Dendranthema zawadzkii</i> (Herbich) Tzvelev	LR	Okrąglica Wąwóz Szopczański	Pleistocene or even late Paleogene steppe relic, gene donor for ornamental chrysanthemums

*Species names after Mirek et al. (2002),

**Threat categories according to Mirek and Zarzycki (2006), Kaźmierczakowa and Zarzycki (2001) and Mirek and Piękos-Mirkowa (2008)

Tab. 2. Results of viability and freezing tolerance tests.

Species	Locality/ year of collection	Germinability test				Freezing test			
		1000 seed weight (g)	germina- tive variant	number of germina- ted seeds	dura- tion (days)	germina- tive variant	germina- bility (%)	number of germina- ted seeds	dura- tion (days)
<i>Dendranthema</i> <i>zawadzkii</i>	Okrąglica/ 2011	0,400	H ₂ O	94	47	12	LN ₂	97	73
							st. LN ₂	97	73
							K	99	74
	Sobczyński Valley/ 2011	0,310	H ₂ O	76	38	16	LN ₂	68	34
							st. LN ₂	52	26
							K	52	26
<i>Erysimum</i> <i>pieninicum</i>	Czorsztyn, Góra Zamkowa / 2010	0,362	H ₂ O	8	4	42	-	-	-
		GA ₃		0	0	42	-	-	-
	Czorsztyn, Góra Zamkowa/ 2011	0,655	H ₂ O	86	43	7	LN ₂	72	36
							st. LN ₂	78	39
							K	80	40
<i>Minuartia</i> <i>setacea</i>	Okrąglica/ 2010	0,103	H ₂ O	80	40	23	LN ₂	48	24
							st. LN ₂	28	14
							K	70	28
									35

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