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Autecology and *ex situ* growth of *Leontopodium nivale* subsp. *nivale* (*Asteraceae*) from North Pirin marbles (SW Bulgaria)

Abstract

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Leontopodium nivale subsp. *nivale* is a local and disjunct endemic of the central Apennines in Italy and the Pirin Mountains in Bulgaria. The aim of this study is to investigate *in situ* microhabitat specifics and *ex situ* ontogenesis regarding the possible future cultivation and to evaluate hazards for wild populations in conditions of human impact and climate change. *Leontopodium nivale* subsp. *nivale* is stenobiont which is difficult to grow *ex situ* and therefore particularly vulnerable. Its wild habitats and populations in Pirin Mts. should be efficiently protected. The results of our study indicate that the stenobiontic plants such as *Leontopodium nivale* subsp. *nivale* are particularly subject to hazard.

Key words: endemic plant, microhabitat specifics, *ex situ* ontogenesis, conservation strategy.

Introduction

Leontopodium (Pers.) R. Br. is a genus of approximately 30 species with an Asian–European disjunct distribution (Blöch & al. 2010). Several molecular clades of *Leontopodium*, each with morphological integrity have been identified. A distinct one contains the European taxa (*Leontopodium alpinum* Cass., *L. nivale* (Ten.) Hand.-Mazz.). These taxa belong to the type section *Leontopodium* [*Alpina* Hand.-Mazz.]. They are genetically distinct from all Asian species in the ITS analysis, although perhaps not as distinctly separated as might be expected from the geographic distance (Blöch & al. 2010). *Leontopodium nivale* is the accepted name Euro+Med PlantBase (2011). *Leontopodium nivale* subsp. *nivale* is a local and disjunct endemic of the central Apennines in Italy and the Pirin Mountains in Bulgaria whereas *Leontopodium nivale* subsp. *alpinum* (Cass.) Greuter or known by the basionym *L. alpinum* Cass. grows in the Pyrenees, throughout the Alps, the Carpathians and the Balkan Peninsula, (Meusel & Jaeger 1992; Euro+Med PlantBase 2011). It is recommend recognising *Leontopodium* *alpinum* and *L. nivale* at species rank until more sophisticated population-level analyses may clarify their status (Blöch & al. 2010). At this stage of AFLP analysis, the populations of Edelweiss from Bulgarian mountains still remain an unsolved taxonomic and phylogenetic issue and no conclusion is possible yet on the relationship between them. AFLP analysis showed that a group containing both European taxa *L. nivale* subsp. *alpinum* and *L. nivale* subsp. *nivale* is indeed well separated from all other 14 taxa in the other groups with a BS of 99%. (Safer & al. 2011, see Fig. 3 Neighbor-net network, highlighting groups and subgroups among species of *Leontopodium*: ACH, *L. alpinum* [Switzerland]; ABG, *L. alpinum* and *L. nivale* [Bulgaria]).

Species delimitation in *Leontopodium* seems to be complicated by the possible occurrence of apomixis. However this phenomenon is poorly investigated in *Leontopodium*. The only species where apomixes, namely diplosporia, has been studied and found is in *Leontopodium nivale* subsp. *alpinum/L. alpinum* (Erhardt 1993; Noyes 2007; Blöch & al. 2010). *L. alpinum* do have apomictic biotypes but more often it is facultative apomixs and sexual reproduction dominates (Hörandl & al. 2011). Additionally along with hermaphrodite plants occur ginomonoecious or andromonoecious ones and in different parts of the range the ratio varies. The population in the Alps is almost entirely andromonoecious (Erhardt 1993). *L. alpinum* in the Tatry mountains is polyploid with $2n= 4x= 52$ (Murín & Paclová 1979; Hörandl & al. 2011) but no data are available for the chromosome numbers of other populations of this taxon as well as for *L. nivale* subsp. *nivale* from Pirin Mts or from the Apennines.

Edelweiss grows in Bulgaria on the marbles of North Pirin Mts. and on the limestone of the Central Stara Planina Mts. (Fig. 1). *Leontopodium nivale* subsp. *nivale* is a perennial herbaceous plant. Stems (1)5–20(30) cm high, erect, simple. Leaves alternate, entire, spathulate, 1.5–4 cm long, densely white lanate; the upper subtending the capitula and equal in length. Capitula subglobose. Involucre 4–6 mm; outer involucral bracts lanceolate, lanate, acute, margin brownish at apex. Florets yellowish white. Achenes 0.5 mm long. *L. nivale* subsp. *nivale* is the taxon that occurs on the marbles of Nort Pirin (Fig. 1). These are plants with patent hairs, stems 1-5 (10) cm and both sides of the leaves lanate. The leaves beneath anthodia, slightly longer then them, spathulate. *Leontopodium nivale* subsp. *alpinum* grows in Stara Planina Mts. (Fig. 1). These are plants with accumbent hairs, stems longer than 5 cm and the upper side of the leaves greenish. The leaves beneath anthodia, much longer then them, oblong-linear (Kuzmanov 2012).

The two species native to Europe, *L. alpinum* (*L. nivale* subsp. *alpinum* known as the common ‘Edelweiss’) and *L. nivale* (*L. nivale* subsp. *nivale*), are part of the cultural heritage of the people living there (Safer & al. 2011). The Alpine Edelweiss (*L. nivale* subsp. *alpinum*) has a long tradition in folk medicine (Safer & al. 2011). References from the year 1582 mentioned the use of Edelweiss for the treatment of diarrhoea and dysentery (Tabernaemontanus 1582). Several other applications for extracts and plant parts of Edelweiss have been described throughout the years, and recent phytochemical research has resulted in the detection of unknown and uncommon secondary metabolites, some with strong anti-tumour and anti-inflammatory biological activities, and they promote cholesterol efflux etc. (Comey & al. 1997; Hook & Sheridan 2001; Stuppner & al. 2002; Dobner & al. 2003a, 2003b, 2004, Dweck 2004; Schwaiger & al. 2004, 2005; Speroni & al. 2006; Hornick & al. 2008; Reisinger & al. 2009; Tauchen & Kokoska 2016; Wang & al. 2016).

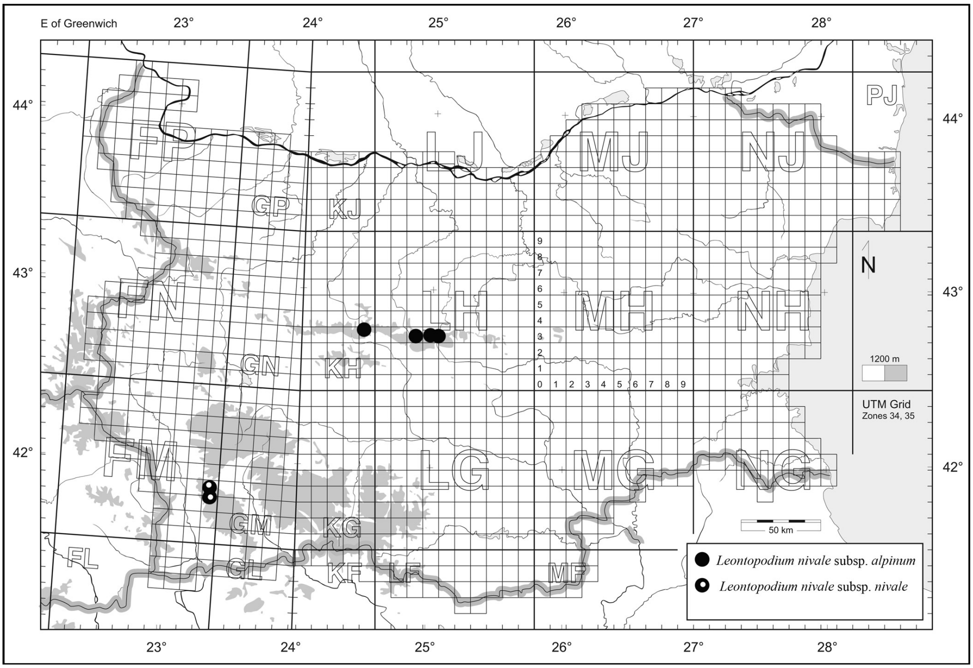

Fig. 1. Distribution of *Leontopodium nivale* subsp. *nivale* in Bulgaria.

The anti-inflammatory activity of the Edelweiss is related to the biosynthesis of leukotrienes, which are potent inflammatory mediators which may have a role in inflammatory diseases such as allergic rhinitis, inflammatory bowel disease and asthma (O'Donnell 1999; Ganzera & al. 2012). These promising results have increased the interest in Edelweiss for pharmaceutical research. There is some in vitro cultivation (Hook 1993, 1994; Zapartan 1996; Butiuc-Keul & Deliu 2000; Trejgell & al. 2010; Daniela & al. 2012).

The Alpine Edelweiss (*Leontopodium nivale* subsp. *alpinum/L. alpinum*) is an important and widely known plant which is now established in cultivation. Edelweiss flowers are highly prized and were collected from the wild in the past, but collection is now regulated or banned in many European countries. This plant is threatened in several European countries and populations declined due to collection in the past; however, it has a large distribution and without information on the extent of population declines, it does not qualify for a threatened category in Europe or the EU 27. It is therefore listed as Least Concern (Khela 2013). Bulgaria is one of those European countries where populations have declined. The IUCN status of *L. alpinum* is Endangered (Bancheva 2011) and the plant is protected by the Biological Diversity Act. Therefore it is important to maintain an appropriate conservation strategy and develop a cultivation establishment.

The aim of this study is to investigate *in sit*u microhabitat specifics and *ex situ* ontogenesis of the endemic *Leontopodium nivale* subsp. *nivale* regarding the possible future cultivation and to evaluate hazards for wild populations in conditions of human impact and climate change.

Materials and methods

Study sites and habitat investigations

The *in situ* field investigations were conducted in the marbleized karst regions of North Pirin Mts., namely the main watershed of North Pirin Mts. with its highest peaks and their slopes build of marble. The study sites are summarized in Table 1 and Fig. 2. They are all located in marble areas of the mountain since *Leontopodium nivale* is calciphilous. The period of investigations of wild populations was during the summers of 1995, 1996, 2001, 2002, 2005, 2014 and 2015. The *ex situ* experiments and observations were conducted during the period 2006-2010. The exact geographic location of all sites was determined in 1995, using a global positioning receiver Garmin GPS 12, Datum WGS 1984, UTM projection (Fig. 2, numbers marking the study sites correspond to the way points recorded with GPS receiver for this plant species in the field). Elevation was double checked with an altimeter (Table 1). Slope and exposure were recorded and described both in the field and using the global positioning system (GPS) methods. Soil samples (two samples at each study site) were taken from the rooting zone of study plants. Each sample was taken from area of 20-30 cm^2 and 4 cm depth. The soil characters were measured after a standard methodology at the Newcastle University in January 2002. The volume of 10 cm^3 of air dry soil (scoop filled and struck of level without tapping) was weighted on digital scales. The soil was ground to pass 2 mm mesh sieve. We transferred 5 cm^3 sieved soil into a bottle and added 100 cm^3 sodium bicarbonate reagent of pH 8,50 at 20°C to extract the phosphorus (Table 2). The concentration of the blue complex produced by the reduction, with ascorbic acid, of the phosphomolibdate formed when acid ammonium molybdate reacts with phosphate was measured spectrophotometrically at 880 nm. The number of μg of phosphorus equivalent to the absorbances of the sample and the blank determinations were calculated from the standard graph. The difference was multiplied by 100 to obtain the quantity of extractable phosphorus in the soil [mg P/kg soil]. Soil pH characters were measured using a pH electrode and meter at about 20°C (between 20,2°C and 20,8°C for each sample). Phytocenosis was recorded after Braun-Blanquet system. The plants were identified and named after Jordanov (1963-2011).

Table 1. Study sites and altitude.

Local Topographic name	Study sites Way point	Altitude
Above Kasan Shelter	7	2605 m a.s.l
Above Kasan Shelter	8	2605 m a.s.l
Below Kasan Shelter	21	2251 m a.s.l
Zhultite skali, Okadenski cirque	16	2170 m a.s.l
Zhultite skali, Okadenski cirque	37	2210 m a.s.l

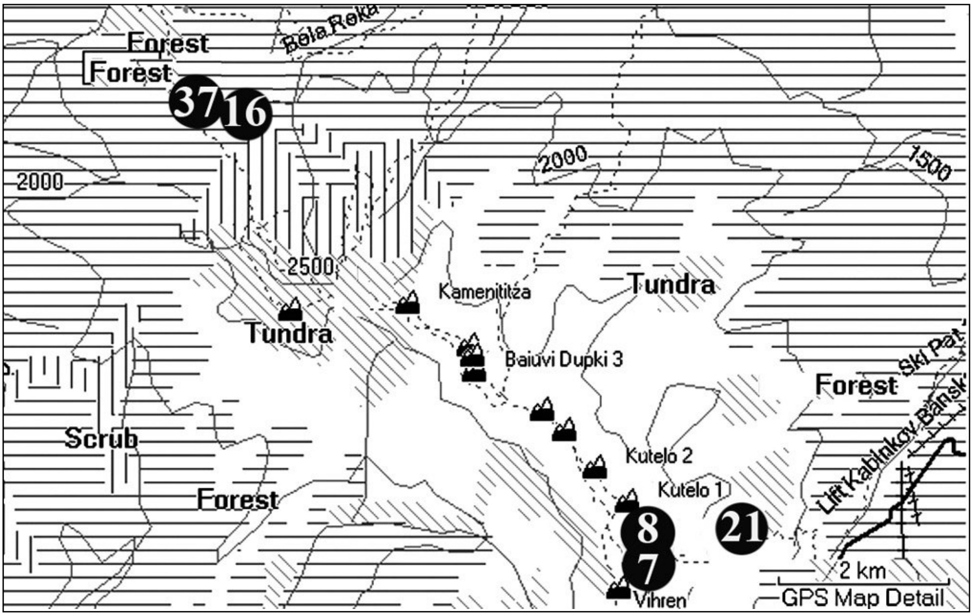

Fig. 2. Localization of the study sites.

Table 2. Features of the soil samples. Legend (-) absent in the sample, (+) sporadic or small, (++) plenty of material, (+++) dominates.

Locality	Sample number	Slope	Exposure	Weight volume [g/10cm^3]	Roots & straw	Sand	Pebbles	Water absorbtion	pH	pH measured at temp°C	mg P/kg soil
Above Kasan Shelter	8	20°	NW	6,3	+	-	+++	medium	7.69	20.5	21.35
Above Kasan Shelter	8	20°	NW	6,3	+	-	+++	medium	7.26	20.5	23.02
Below Kasan Shelter	21	35°	E	7,8	+	++	+	delayed	7.70	20.6	20.65
Below Kasan Shelter	21	35°	E	8,4	+	++	+	delayed	7.42	20.7	23.89
Zhultite skali	16	33°	SE	10,8	++	++	+	medium	7.29	20.5	20.95
Zhultite skali	16	33°	SE	10,8	++	++	+	medium	7.55	20.7	20.72
average									7.49	20.58	21.76
stdev									0.193	0.098	1.361
min									7.26	20.50	20.65
max									7.70	20.70	23.89

Fig. 3. *Ex situ* experimental plot in the vicinity of Dobrinishte in the foothill of Pirin Mts. – drainage layer and stonework with marble peaces.

Ex situ *experiments and observations*

Some local people in the settlements in the northern foothills of Pirin Mts (Bansko and Dobrinishte) are interested in growing Edelweiss in their gardens. They tend to transplant adult plants from the native populations mostly. Therefore our *ex situ* experiment was in two steps – transplantation of adult plants and growing seedlings. Firstly we obtained permissions from MOEW - Bulgarian Ministry of Environment and Water No: RD – 744/15.07.1/2005 and No: 67/20.06.2006.

The experimental plot for adult transplants was established in 2005 in the yard of a house in Dobrinishte village, in the foothill of Pirin Mts. The experimental rock garden for seedlings was established in 2006 near the village, in a hay meadow of approximately 500 m^2 near the river, situated at 865 m above sea level, at N 41°48’80,9” and E 23°33”67,4” (WGS84). We chose the steepest part of a hay meadow with an exposure to the north-east. The place was chosen with consideration for several factors: i) close enough to the river for watering; ii) away from potential floods; iii) moderately shaded; iv) the snow lies relatively long here, protecting the plants from the spring frosts, and providing a cool microclimate in summer (Fig. 3).

Three adult plants were carefully excavated from their native habitats and immediately transplanted in the experimental plot. We used marble peaces with holes drilled in them to resemble as much as possible the natural environment. The soil was predominantly from the native habitat.

Seed was collected in September 2005 and kept cool (4°C, in the fridge, but not frozen). Several sets of seeds were processed for germination on moisture filter paper at natural light – dark photoperiod and average temperature 19°C. Once the seeds had germinated, the seedlings were transferred individually to plastic pots filled with a mix of 30% rough marble sand, 30% sieved humus, 30% sieved good soil, 10% perlite or with a mix of 60% silty brown soil and 40% rough marble sand. When seedlings were at the 2-6 true leaf stage, they were transported to the experimental rock garden. They were planted singly into the flower beds with fine marble gravel top-dressing. We followed the concept of discrete description of plant ontogenesis (Komarov & al. 2003)

Data analysis

Descriptive statistics was used to analyze the data obtained.

The comparative analysis of the vegetation at the sites was done calculating the Jaccard coefficient (Muller-Domboa & Ellenberg 1974). The Jaccard coefficient measures the similarity of two sample sets (Table 3). It uses the ratio of the intersecting set to the union set as the measure of similarity. Thus it equals zero if there are no intersecting elements and equals one if all elements intersect.

$$T = \frac{N_c}{N_a + N_b - N_c}$$

Where: *Na* - number of elements in set A, *Nb* - number of elements in set B, *Nc* - number of elements in intersecting set.

A comparative analysis of the climate parameters both in native habitats and in the *ex situ* experimental plot was performed based on data officially available from national institutions and local meteo-stations (Table 4). The measurements in Vihren hut (1954-1974) and Bansko (1933-2014) were conducted by the National Institute of Hydrology and Meteorology of Bulgaria. The measurements at Vihren hut (2010-2015) were carried out by Grunewald & al., while those at Treeline location close to the *in situ* study sites (native habitats) by Panayotov & al. (Grunewald & al. 2016).

Table 3. Jaccard coefficient of similarity between the plant communities of the investigated subpopulations of *Leontopodium nivale* subsp. *nivale* (N=5)

Study sites	Way point 8	Way point 7	Way point 21	Way point 16
Way point 8				
Way point 7	0,32			
Way point 21	0,69	0,37		
Way point 16	0,26	0,64	0,58	
Way point 37	0,47	0,31	0,73	0,78

Results

Autecology of Leontopodium nivale *subsp.* nivale

Leontopodium nivale subsp. *nivale* grows in North Pirin Mts in the belt of the subalpine meadows (study sites 16, 21, 37) and in the alpine belt (7, 8). The altitude of the microhabitats ranges in wide diapason 2170-2605 m a. s. l. (Table 1). The exposure of the microhabitats is diverse so the plant is tolerant to this factor.

The granulometric composition of the soils, determined by sieving them (2 mm mesh sieve) revealed that the soils consisted of rough particles-sand and pebbles (Table 2). Soil pH in our samples was neutral to slightly alkaline (Table 2) and this confirmed the calciphilous nature of the Edelweiss. Extractable phosphorus (P) in the soil was between 20.65 g/kg soil (site 21, Table 2) and 23.89 (site 8, Table 2).

In most of our study sites *Leontopodium nivale* subsp. *nivale* grows as a calciphilous chasmophyte together with *Potentilla apennina* subsp. *stojanovii* Urum. & Jáv. and *Dryas octopetala* L. It occupies habitats where the vegetation cover varies between 50% and 80%. In our study sites *L. nivale* is an element of plant communities dominated by *Sesleria korabensis* (Kumm. et Javorka) Deryl, and *Carex kitaibeliana* Degen ex Bech. Highly abundant are *Potentilla apennina* subsp. *stojanovii* Urum. & Jáv., *Dryas octopetala* L., *Saxifraga ferdinandii-coburgii* Kellerer et Sund, *Saxifraga oppositifolia* L., *Campanula cochleariifolia* Lam., *Veronica kellererii* Degen & Urum., *Linum capitatum* Schultes, *Acinos alpinus* (L.) Moench, *Centaurea achtarovii* Urum., *Papaver degenii* (Urum. & Jáv.) Kuzmanov, *Alyssum cuneifolium* Ten., *Thymus perinicus* (Velen.) Jalas, *T. thracicus* Velen., *Helianthemum nummularium* (L.) Miller, *Rhodax canus* (L.) Fuss., *Cerastium alpinum* L., *Aster alpinus* L., *Achillea ageratifolia* (Sibth. et Sm.) Boiss., *Daphne velenovskyi* Halda, *Oxytropis urumovii* Jav. and *O. kozurahovii* Pavolova, Dimitrov & Nikolova. The floristic compostion of the plant communities in our study sites demonstrated more or less high similarity (Table 3). Interestingly, similarity was higher between sites that were not in the near vicinity (e. g. way points 7 and 16) and lower between closely situated sites (e. g. way points 7 and 8).

Ex situ *ontogenesis of* Leontopodium nivale *subsp.* nivale

Transplantation of adult plants - All three plants that we transplanted in September 2005 survived the winter and leaf rosettes were developed in April 2006. One of the plants

Table 4. Climate parameters measured close to the *in-situ* locations and the *ex situ* experimental site.

Location & altitude, m a.s.l.	Period	Para-meter	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Yearly
Vihren hut	2010-2015	T mean, °C	-3.5	-3.2	-1.5	2.7	7.7	11.3	14.0	14.4	8.6	5.5	4.3	-2.1	4.9
1950 m a.s.l.		T min, °C	-19.8	-17.3	-13.3	-11.3	-1.4	0.3	3.8	3.9	-1.4	-8.9	-10.8	-18.2	-19.8
		T max, °C	10.1	11.8	10.1	17.6	23.9	31.6	33.6	31.6	21.3	19.4	26.0	10.9	33.6
		H, %	64.3	63.1	68.5	67.5	74.3	68.3	66.3	64.3	75.0	65.6	64.2	63.7	67.1
Vihren hut,	1954-1974	T mean, °C	-4.7	-4.2	-2.5	0.8	5.8	9.8	11.9	11.7	8.3	4.5	1.2	-2.7	3.3
1950 m a.s.l.		Precip., mm	146	144	121	107	123	112	81	58	86	109	173	175	1435
		T min, °C	-23.2	-23.5	-19.7	-14.5	-8.1	-5.5	-1.2	-0.5	-6.5	-8.7	-16.3	-22.5	-23.5
		T max, C	9.3	11.4	11.4	14.1	19.5	24.0	25.2	25.8	24.4	18.5	15.1	14.6	25.8
Treeline,	2013-2016	T mean, °C	-3.0	-1.7	-1.5	2.5	5.8	9.8	13.0	12.8	9.1	5.8	2.7	-0.6	4.6
2250 m a.s.l.		T min, C	-18.4	-18.3	-12.3	-11.0	-3.1	1.0	4.5	3.3	-1.5	-7.8	-10.2	-19.4	-19.4
		T max, °C	4.2	6.5	3.8	10.0	10.4	17.7	15.6	14.4	14.2	8.9	7.0	4.2	17.7
Bansko,	1933-2014	T mean, °C	-1.6	0.1	3.7	8.7	13.4	17.0	19.1	18.8	14.9	9.7	4.6	0.1	9.0
936 m a.s.l.		Precip., mm	60	58	50	56	60	53	40	32	37	59	73	77	656

bloomed in July 2006 (Fig. 4A). The plant developed one flowering stem and the anthodia were only three and small but they produced seeds (achenae, Fig. 4B and 4C). The second winter period was survived by only two individuals and in June 2007 the full bloom was recorded. One of the plants developed three flowering stems and the other – nine flowering stems (Fig. 4D). The flowering stems were big with 6-9 large anthodia. Both plants overwintered successfully and developed leaf rosettes. The plant that previous year bloomed lavishly formed a flowering stem (Fig. 4E). By August 2008 both plants dried and vanished despite the regular moderate watering. In summary, during the first season after transplantation the flowering was depressed. Lavish flowering was achieved the second season but the plants did not live long enough for a third flowering. The breaking point was the summer. Our experiments revealed that Pirin Edelweiss adult plants have weaker ability of adaptation to *ex situ* conditions compared to other rare and endemic species which we transplanted in the same experimental plot such as: *Aubrieta gracilis* Boiss., *Veronica kellereri* Degen & Urum., *Hypericum linarioides* Bosse, *Cerastium decalvans* Schloss. & Vuk., *Achillea chrysocoma* Friv., *Thymus thracicus* Velen. All these plants demonstrated

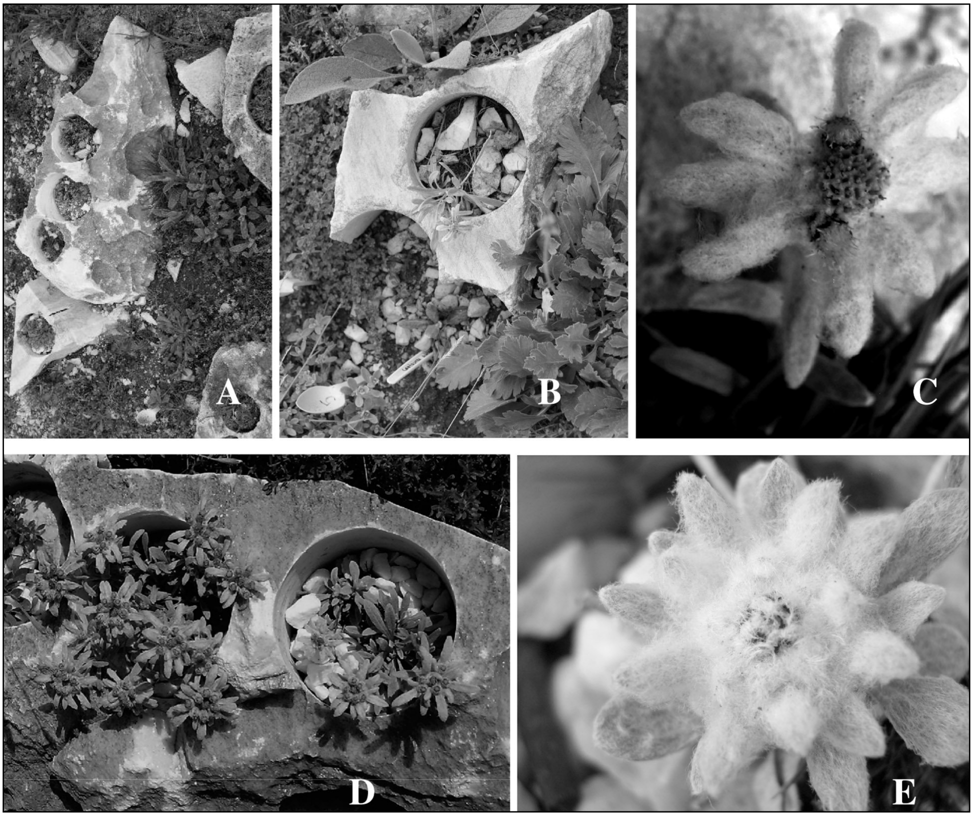

Fig. 4. Adult plants - transplantation and ontogenesis. A: 2006 April, beginning of vegetation; B: and C: 2006 July full bloom; D: 2007 June full bloom; E: 2008 June beginning of blooming.

ability for vegetative propagation, enlargement of the biomass and lavish flowering.

Among the possible reasons for the mortality of the transplanted plants were unfavorable climate conditions during the experimental years. 2007 especially was characterized by unusual climate conditions – the winter was generally dry with twice as low a precipitation sum compared to the long-term (1933-2014) average for Bansko station (Table 4), combined with the third highest winter temperature. Especially warm were January and February, for which the warmest average temperature was recorded. Additionally the summer was also unusually warm with the fourth highest June-August temperature for the record. Despite the fact that the plants were watered during the summer, the combination of winter draught and high temperatures plus summer high temperatures might have created stress conditions that made the plants vulnerable to pathogens.

Seed propagation - *Leontopodium nivale* subsp. *nivale* from Pirin Mts. reached a germination percentage of 85.4 ± 7.6% (average ± SE) during the first two years after the seed collection under variable conditions (5-28°C; 13/11 and 14/10 photoperiod; no GA3 addition, Table 5). Survival of the seedlings in the pots until the moment to be planted in the rock garden was better when seeds germinated at higher temperature and with a longer light photoperiod (in May). Germination percentage with GA3 addition was 80% (Table 5). The untreated processed for germination on moisture filter paper at natural light – dark photoperiod and average temperature 19°C seed of Edelweiss - germinated well (71%-89%). The seedling developed well at the early stages (1-4 real leaves and size 3-5 mm). Later on many of the seedlings planted in the experimental rock garden did not survive (Tables 5 and 6). None of the plants grown by seedling survived for five years i. e. during the whole experimental period. During the first year after seed collection, 80 % of the experimental set of seeds germinated (Table 5). For a period of four months (February to July) seeds kept germinating gradually. Interestingly there was no moulding of the moisture paper around the seeds. All seedlings survived to be planted in the experimental rock garden, but only half of the seedlings planted *ex situ* survived till the end of the summer (Tables 5 and 6). Only one plant grown by seed survived for four years (Table 6). During the second year after the seed collection germination remained very high. Germination was similar both for those stimulated with giberellic acid (GA3) and for non-treated seeds (Table 5). Also survival of the seedling resulting by both regimes of germination was similar. However seeds processed for germination later in spring at higher temperature germinated better (Table 5). These seedlings survived better as well (Table 5).

During the forth year after the seed collection germination remained very high as in the previous years (Table 5). Survival of the seedlings to the stage for planting in the rock garden however was poor (Table 5). The germination experiment set in December revealed that seeds dormancy is breakable in the autumn-winter period if the temperature regime is favorable (Table 5). The seedlings did not survive. Consequently four years period reduces the seeds viability. Percent of germination decreased twice for period of seven years (Table 5). Almost half of the seedlings (42%) grown in the first year after seed collection survived in the rock garden during the whole summer (Table 6). The survival of seedlings grown during the second year after the seed collection was less successful (20%). During their first summer seedlings passed through the stage of juvenile plants, and reached the stages of immature and virginal plants. The reproductive stage was reached by some plants during

Table 5. Germination of Edelweiss seeds and survival of the seedlings at their early stages.

Year after seed collection	Processed for germination	Period for germination	Experimental set of seeds	Temperature during germination period	Percent of germination	Survived to be planted in the rock garden
I	2006 February	Four months	30	5-28°C	80%	24
II	2007 March non-treated	Three months	22	12-22°C	82%	2
	March treated with	One month	45	12-22°C	80%	6
	GA3 May non-treated	One month	85	19-28°C	98%	66
	Total		152	12-28°C	Average 87%	74
IV	2009 Second half of April to Second half of May	One month	45	16-28°C	82%	1
IV	2009 December	One month	45	22,5°C	71%	71%
VII	2013 Second half of January to Second half of February	One month	31	22,5°C	35%	35%

the second or third summer (Table 6). They developed 1-4 flowering stems. After that, many of them died (Table 6). Most vulnerable are plants in their juvenile-immature stages. Most critical for adult plants (virginal and reproductive) is the winter period.

Both adult transplants and plants grown from seed *in situ* had different habitus compared to wild plants. They were obviously bigger (stems 5-9 cm, leaves of the rosettes 5-6 cm and the rosette of bracts 3-4.5 cm) and this was valid particularly for the plants grown from seed *in situ*. However they preserved all other diagnostic features for the taxon (patent hairs, both sides of the leaves lanate, bract rosette leaves spathulate), except the size. *Ex situ* grown plants had patent hairs, and both sides of the leaves had silvery whitish hairs. The leaves beneath anthodia remained slightly longer compared to the anthodia, spathulate.

The temperatures at the experimental plot were higher (Table 4) because of its position at a lower altitude compared to the wild populations. This had an impact on the flowering phenology, which was about a month earlier compared to the natural habitats no matter whether these were transplanted adults or plants grown by seed. For the young seedlings the juvenile and immature stages were critical and 50% to 68% mortality was recorded during the summer after their planting in the experimental plot. The winter was Rubicon for all mature seedlings and regardless of whether they were at the virginal or reproductive stage 50% to 60% did not sur-

Table 6. Seedlings planted in the experimental rock garden - ontogenesis and survival.

		Reading of ontogenesis and survival of plants grown form seed		
		2006 planting in May	2007 planting in April	2009 plating in May
2006		First year		
	May	24 juvenile		
	August	12 immature		
	October	10 virginal		
2007		Second year	First year	
	April	6 virginal	74 juvenile	
	May	6 virginal	24 immature	
	August	5 virginal	14 virginal	
2008		Third year	Second year	
	May	1 reproductive	14 virginal and reproductive	
	June	1 (1 reproductive blooming)	14 (4 reproductive blooming)	
	August	1 (1 reproductive fruiting)	14 (4 reproductive fruiting)	
	September	1 (1 reproductive fruiting)	14 (4 reproductive fruiting)	
2009		Forth year	Third year	First year
	May	1	7 (3 reproductive blooming)	1 juvenile
	June	1 (1 reproductive blooming)	7 (3 reproductive fruiting)	1 immature
	July	1 (1 reproductive fruiting)	7 (3 reproductive fruiting)	1 virginal
2010		Fifth year	Third year	Second year
	July	0	4 (reproductive, no blooming)	1 virginal

vive (Table 6). An explanation could be found in the combination of winter warm periods which cause thinner snow cover that is not permanent the whole winter. The plants unprotected by snow are exposed to sporadic frosts. Also the summer high temperatures might have created stress conditions that made the plants vulnerable to pathogens.

Discussion

Leontopodium nivale subsp. *nivale* population in Pirin Mts. grows as a calciphilous chasmophyte on neutral to slightly alkaline soil with poor morphology and quality. The studied populations are localized in the criolithogenic belt. Ninov (1982) has described well this extremely poor soil-forming process and scanty soil hidden between the marble boulders, rock cracks, grooves, and fissures as a result of the high elevation with harsh climate and marble terrain. The periglacial relief is a result of crionivalic processes with periodical freezing and unfreezing of the soil and the weathering crust. Due to the karst terrain no ponds are formed because water is drained. Regeneration processes are slow, so that the average vegetation cover is about 50%. In result the soil is primitive, poorly developed and its cover is rather loose. The nature of the marble weathering is the reason for the fragmented soil cover, poor development and functioning as well as poor interaction with the vegetation. The index of the potential bio-production is 0,1 – the same as in the tundra or in the desert. Thus most of the soils here have poor morphology and quality. They have a ”cryo” temperature regime and are defined as Cryrendolls. Such soils are rare for Bulgaria. They occur only here in Pirin and rarely in Slavyanka Mts. (Ninov 1982). Being formed on hard rock they are defined in details, at a lower taxonomic level as Lithic Cryrendolls (ST) or Lithic Leptosols – Rendzic Leptosols (F.A.O.).

The calciphilous chasmophyte *Leontopodium nivale* subsp. *nivale* in Pirin Mts. grows together with *Potentilla apennina* subsp. *stojanovii, Dryas octopetala,* as well as with *Carex kitaibeliana, Saxifraga ferdinandii-coburgii* and *Campanula cochleariifolia. Leontopodio - Potentilletum stojanovii* in a plant community described for Pirin Mts (Mucina & al. 1990) and it is noted as a specific one. The native populations of *Leontopodium nivale* subsp. *nivale* occupy habitats which belong to montane tall-herb, grassland, fell-field and snow-bed vegetation. These are alpine and subalpine open calcicolous herbaceous and alpine calcicolous herbaceous communities near melting snow-patches. Here psychrophytous and cryophytous hecistothermal vegetation is found in the alpine woodless belt; calciphilous cryophytous grass formations *Kobresieta myosuroides, Cariceta kitaibelianae, Seslerieta korabensis* and small shrub formations *Dryeta octopetalae, Saliceta reticulatae,* etc. (Bondev 1991). Lately *Leontopodio nivalis-Elynion myosuroidis* (Blasi & al. 2003) Di Pietro & Mucina (Crytrý & al. 2015) is described as a new alliance of Southern Europaean alpine tundra. This is an important syntaxonomic description, because if an Apennine-Balkan delimitation of the *Leontopodio-Elynion* was accepted, the mountain ranges of Korab, Prokletije, Rila and Pirin would represent the southernmost limit of the *Carici-Kobresietea* in south-eastern Europe (Chytrý & al. 2015). Adaptations to habitat specifics might be related to endemism. For example, the main environmental variable discriminating sites occupied by two butterworts in the Alps is the elevation. Growth and reproductive performances of the wide spread *Pinguicula vulgaris* and the endemic *P. arvetii* are influenced by the site conditions. The endemic is typically confined to the metamorphic rocks of the Pennidic domain where the “Calcescisti e Pietre Verdi” complex is largely predominant. From the phytogeographical viewpoint, it belongs to a floristic contingent centred in the south-western Alps (Zaccara Bertolini & al. 2016).

Leontopodium nivale subsp. *nivale* occurs in two NATURA 2000 habitats: 6170 Alpine and subalpine calcareous grasslands and 8120 Calcareuos and calcshist screes of the montane to alpine levels (Tzonev & al. 2009; Roussakova 2009, 2011).

Our experiments revealed that adult individuals *L. nivale* subsp. *nivale* have weak vegetative propagation ability and low potential for transplantation *ex situ*. Consequently the result after transplantation is gradual extinction. Therefore transplantation of adult individuals for further *ex situ* propagation is not a prospective approach for cultivation. The local people in Bansko and Dobrinishte in the foothill of Pirin Mts. who tend to transplant adult Edelweiss individuals in their gardens should be discouraged from doing that.

The optimal germination of *Leontopodium nivale* subsp. *nivale* from Pirin Mts. was two years after the seed collection, 85.4 ± 7.6% (average ± SE) under variable conditions in spring (5-28°C; 13/11 and 14/10 photoperiod; no GA3 addition). The Edelweiss achenae germinate well if they are exposed to the sun light and on the surface of the soil. They need a high air temperature (18-20°C) and longer light photoperiod. Such conditions are favourable for many members of family *Asteraceae* (Schlorhaufer, pers. comm.). *L. nivale* from the Apennines (Italy) reached a germination percentage of 98.0 ± 2.0% (average ± SE) under control conditions such as 20°C,12/12 photoperiod and without GA3 treatment (Di Martino & al. 2014). The two disjunct populations from Pirin Mts. and the Apennines demonstrated similar germination behaviour. The optimal temperature for germination of *L. nivale* subsp. *alpinum* seeds is 25°C (RBG Kew, Wakehurst Place, http://data.kew.org/sid/SidServlet?ID=13572&Num=B6i).

Seed viability of *L. nivale* from Pirin Mts. decreased for 4-7 years period. Seeds of *Leontopodium nivale* subsp. *alpinum* (*L. alpinum*) can be maintained for 3 years in commercial storage conditions (Priestley 1986). During their first summer seedlings reached the stages of immature and virginal plants. Some plants reached reproductive stage during the second or third summer. Seedling survival was poor in all experimental regimes. Most critical was the winter period. Most vulnerable were plants in their juvenile-immature stages.

Morphological characters – patent hairs, both sides of the leaves lanate, bract rosette leaves spathulate – by which *Leontopodium nivale* subsp. *nivale* is distinguished (Kuzmanov 2012), proved to be genetically determined and preserved when plants were grown *ex situ*.

The Pirin Edelweiss is stenobiont which is difficult to grow *ex situ*. Similar results are obtained in Romania (Lidia 2012). *Leontopodium nivale* subsp. *alpinum* is one of the taxa from spontaneous flora which does not resist in the conditions of rockeries, “Anastasie Fătu” Botanical Garden together with *Campanula carpatica* Jack, *Dryas octopetala* L., *Minuartia laricifolia* (L.) Schinz ex Thell (Lidia 2012). Our preliminary data for *ex situ* growth of *D. octopetala* demonstrate similar discouraging results (unpubl.). At the same time, Alpine Edelweiss (*L. nivale* subsp. *alpinum*/*L. alpinum*) is now established in cultivation (Khela 2013) which may indicate better tolerance of the populations from Central Europe.

Serious threats for the wild populations of Edelweiss (*Leontopodium nivale* subsp. *nivale* and *L. nivale* subsp. *alpinum*) are adaptation to specific habitat conditions, low numerical strength of most populations, fragmentation of the distribution area, low reproductive potential destruction of individuals for commercial purposes and by tourists (Bancheva 2011). Our *ex situ* experiments revealed that *L. nivale* subsp. *nivale* is particularly stenobiontic and therefore especially vulnerable. Its wild habitats and populations in Pirin Mts. should be efficiently protected. It is crucial to consider the difficulties for *ex situ* growth in the future Management Plans for National Park Pirin Mts. with respect to the concept of sustainable development.

The climate in the natural habitats is characterized by average yearly temperatures close to 5ºC with negative monthly temperatures in the December-March period. The warmest period is July-August (Table 4). The absolute maximum measured temperature was 33.6 ºC, but this is in a location of about 200 vertical meters below the natural locations of the studied species. The absolute minimum temperature was -23.5 ºC. We note that temperatures recorded during measurements in the last decade were higher than temperatures recorded in the period 1954-1974, which follows a general trend of increase of temperatures in the high Bulgarian mountains (Grunewald & al. 2009; Nojarov & al. 2012). The data from Mussala peak station (2925 m a.s.l.), which is the only long-term reliable high-mountain record in the region, shows a trend of temperature increases with continuously positive temperature anomalies relative to the 1961-1990 period after the mid 1990s. This trend is well expressed in the summer period, but also in late autumn (Fig. 5). The rainfall is significant (about 1400 mm), 1/3 of which falls in the winter period primarily as snow, forming persistent thick snow cover, which remains about 150-180 days. Although in winter much of the locations of the plants are covered by snow, on rock ridges and steep rocky slopes the snow cover may be very thin and temporary disappearing in winters with less precipitation. Such cases were observed in the last decade when Edelweiss locations were without snow for short periods in some winters.

The climate close to the *ex situ* experimental site is characterized by higher temperatures (average annual temperature is 9ºC) and precipitation (656 mm), which is twice as low (Table 4, Bansko location). In some winters long precipitation-free periods and drought conditions were observed both at the foot of the Mountain Range and the Mountain locations (Panayotov & al. 2010).

The low survivorship in the *ex situ* experimental site, which is characterized by drier and warmer conditions might be a sign of potential future problems for the original populations in case of drier and warmer years. Such are expected under the regional climate change scenarios (IPCC 2013). Climate changes may alter conditions seriously affecting the growth niches of alpine species, thus limiting the distribution of some of them (Grabherr & al. 2010). In such cases especially species found in micro-habitats with already very limited distribution may be those which will be the most threatened. In most cases they are unable to outcompete species with better adaptability and find new habitats when the original ones become unfavorable.

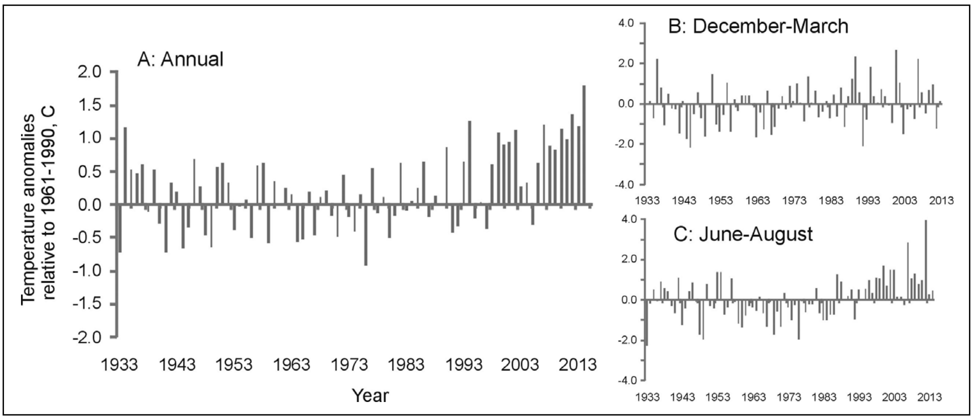

Fig. 5. Temperature anomalies relative to the 1961-1990 period in the record of Mussala peak meteorological station: A) annual average temperatures; B) winter temperatures; C) summer temperatures.

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