Conservation of Endangered Species: *Androsace mathildae* Levier (*Primulaceae*) in Central Italy

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Abstract

Climate change influences the distribution of species, causing the displacement of distribution areas, both in altitude and latitude. The potentially most climate change-susceptible taxa are those of mountain peaks, with limited distribution areas, such as rare or endemic species. In this paper, we deepened the study of *Androsace mathildae* Levier, a plant that lives in Central Italy over 2500 m asl. This species, classified as a rare plant according to the Red List of Plants of Italy, is included in Appendix I of the Bern Convention, Annex II and IV Habitats Directive 92/43 CEE, and is protected under Regional Law n. 45/79 of the Abruzzo Italian Region. In order to preserve *A. mathildae*, morphological observations and *ex situ* preservation have been performed. Results indicate that the germination of seeds increases in the presence of extracts of humic acids obtained by the decomposition of the lower leaves of the plant. The presence of *Pyrenophora bromi* (*Ascomyta*) on the leaves as a decomposition agent, has never been reported either on *A. mathildae* or at these altitudes. A number of experiments to obtain callus induction and neomorphogenesis were conducted to identify suitable procedures for *in vitro* stabilization. The obtained shoots had a high number of lateral buds (6.3 per plant, on average) which were used to increase the number of clones present in the growth chamber within a few sub-culture passages. This *in vitro* material will be used in cryopreservation and to improve the restocking activity of *A. mathildae*.

Keywords

Biodiversity, Alpine Botanical Garden, Morphology, *In Vitro* Preservation

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1. Introduction

EU Habitats Directive 92/43 identified the species of European Community (EC) interest whose conservation requires strict protection (Annex II-IV). Preservation, protection and improvement of the quality of the environment, including the conservation of natural habitats and flora, represent a fundamental general interest objective of the EC.

Conservation of endemic species requires a thorough knowledge of their biological and morphological features in order to define an appropriate strategy. The case study presented herein highlights how conventional strategies of propagation, such as the germination of seeds, do not prove to be efficient without the use of germplasm *ex-situ* conservation techniques.

Androsace mathildae Levier is a glacial relict present on a few stations in the Gran Sasso and Majella Mountains, so it is classified as a rare plant according to the Red List of Plants of Italy, it is further included in Appendix I of the Bern Convention, in Annex II and IV of Habitats Directive 92/43 CEE and it is protected under Regional Law n. 45/79 of the Abruzzo Italian Region [1]-[3].

Alpine plants respond to the harsh environment with a high degree of specialization [4].

A noteworthy aspect in plant survival at high altitude relates to the snow cover, that determines the distribution of plant species in the alpine zone. The snow cover has protective effects for the plants, consisting in the prevention of exposure to low temperatures, winter desiccation, ice blast and solar radiation (potentially dangerous to dormant tissue). However, prolonged life under the snow requires resistance to physico-chemical stresses and snowbed pathogens. In particular, the snow cover affects the phenorhythm of the plants, which need to have sufficient carbon reserves to overcome the prolonged winter and for clonal or reproductive propagation [4]. Vegetative shoot apices and leaf meristems in most alpine plants are buried several centimeters below the ground and thus are not exposed to low and high temperature extremes.

The snow cover and the paucity of humus present in the cracks where *Androsace mathildae* lives cause water stress due to reduced water availability. Alpine plants acquire water and mineral resources from substrates which differ in many respects from those common at lower altitudes. The grain size distribution found in alpine soil depends strongly on parent material.

By avoiding nucleation, a process called supercooling allows leaf and stem tissues to cool below freezing point without freezing. This process is favored by compartmentalization of plant water into cells and vessels, by cell wall impregnation with lignin and by the absence of particles which could initiate the crystallization process [4].

The high capacity of clonal propagation in these high altitude species is due to the adjustments that the plants have developed to protect themselves from frost. In fact, the late frosts at high altitudes endanger the blooms and thus the possibility of sexual reproduction [5]. The compression of live zone and the small scale patterns of life conditions in steep alpine terrain represent natural experiments which provide unbeaten opportunities to study the plant adaptation and the mechanisms for survival of physical stress conditions [6] [7].

The potentially most climate change-susceptible taxa are those of mountain peaks, with limited distribution areas, such as rare or endemic species [8]. The alpine flora is at risk of extinction in the European mountains, especially where there is no possibility of moving to a higher altitude (low and hot mountains, as Prealps and the Northern Apennines). Species occurring in isolated populations at the highest peaks of the Apennines, such as *A. mathildae*, are also at risk [9]. For conservation purposes, the plant is currently maintained as a small number of individuals in the flower beds in the Alpine Botanical Garden of Campo Imperatore. This garden is located in the Gran Sasso massif, within the "Gran Sasso e Montidella Laga" National Park, at 2117 m asl; thus its peculiar location makes it ideal for *in situ* conservation of Central Appennine high altitude species. The living plant collections and, increasingly, seed banks and cryopreserved tissue cultures maintained by botanic gardens, form a significant *ex situ* reservoir of endangered plants [10] [11].

Today several botanical gardens use *in vitro* techniques in germplasm conservation, and long-term storage of material in culture for potential applications of cryopreservation [12].

Androsace mathildae can be found in the cracks of limestone at an altitude between 2500 and 2900 m asl [13]. Its discovery dates back to 1875 and it is ascribed to the Swiss naturalist E. Levier while he was hiking on the Gran Sasso Mountain. He dedicated the plant to his wife Mathilde, since she saw it first, and he performed the early studies on its iconography and botanical characteristics [14].

The genus *Androsace* L., (*Primulaceae*) comprises about 150 species distributed in extra-tropical mountain ranges of the Northern hemisphere. They are particularly common in the temperate-cold regions of the Arctic

and in Asia, North America and Europe (Alps and Pyrenees) [15] [16].

A. mathildae, a European member of *Aretia*, is a nano-chamaephyte present in the higher peaks of the Central Appennines [17] [18]; it is a perennial cushion plant, up to 3 cm high, with single rosettes or forming a cushion with a diameter no larger than 3 - 5 cm. The leaves are basal, lanceolate, shiny green and glabrous except for a few hairs at the leaf tips. The flowers are axillary, single, and the pedicel is often curved when fruiting. The calyx is green, with a stellate pubescence, and the corolla has white petals with a yellow eye. The fruit ripens in late summer and contains from three to seven seeds (generally five), which upon falling end up inside the leaves of the basal rosette or slightly away from it; the flowering time occurs in June-July [19]-[21].

Until recently, it was believed that *Androsace mathildae* was also present in the mountains of the Balkan peninsula (M. Komovi, Montenegro) [22]. The molecular phylogeographical investigations on the European members of *Aretia* by amplified fragment length polymorphism (AFLP) markers revealed a strong genetic divergence between the Italian and the Montenegrin populations. The study led to the establishment of a new species called *A. komovensis* sp. nov., which morphologically resembles *A. mathildae* Levier from the Abruzzo mountains (Italy), but differs in the persistent, dense and regular indumentums of the leaf margin [23]. Furthermore, molecular phylogenetic data indicate that *A. komovensis* is not closely related to *A. mathildae* Levier, but instead it is a sister species to the Eastern Alpine endemic *A. hausmanni* Leyb. [23].

We invested great effort in a comprehensive study about this *Primulacea* in order to describe its morphological and ecological characteristics. A number of experiments to obtain callus induction and neomorphogenesis were conducted to identify suitable procedures for *in vitro* preservation of *A. mathildae*. This *in vitro* material will be useful in cryopreservation and improvement of restocking activity.

2. Materials and Methods

2.1. Plant Material

The material used for the study of *A. mathildae* was collected in late summer from 2008 to 2014 from several stations on Gran Sasso Mountain. Existing stations of *Androsace mathildae* present on the chain of Gran Sasso are: Corno Piccolo (2585 m asl rocks, 7 individuals, 640 m^2); Corno Grande (with two stations: at 2780 m asl, 70 individuals, 925 m^2 and at 2632 m asl, 100 individuals, 432 m^2), Monte Camicia (2490 m asl, 14 individuals, 5 m^2) and Monte Prena (2553 m asl, 2 individuals, 5 m^2) [24] (**Figure 1**). We harvested plant parts (leaves, flowers, lateral buds, roots and seeds) in very small quantities, in order to protect individuals and minimize structural damage. The material was identified using Pignatti's dichotomous analytical keys based on macroscopic and microscopic morphology [21].
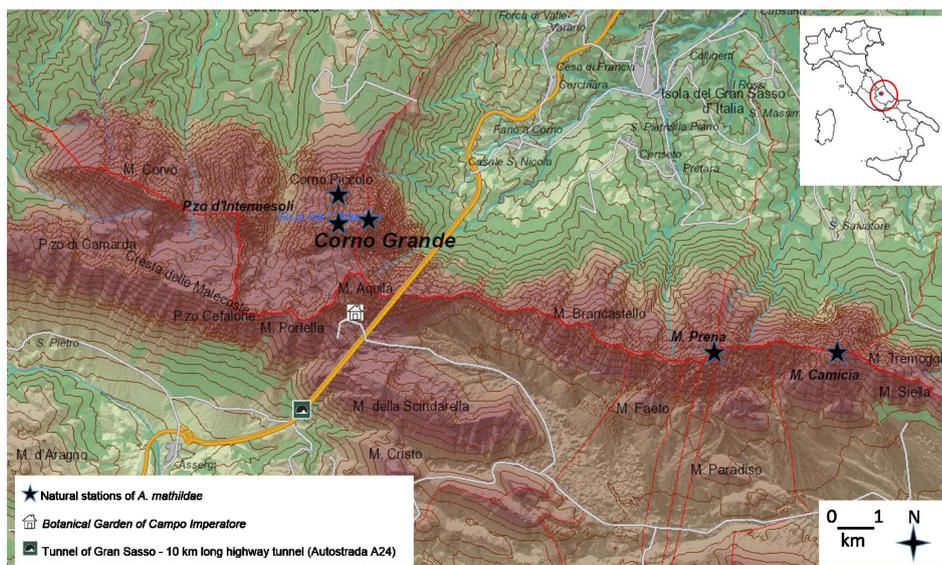
Figure 1. Map showing the chain of Gran Sasso (Italy). The distribution of stations of *Androsace mathildae* is indicated with black stars.

2.2. Morphological and Cytological Analysis

The morphology of the various parts of *A. mathildae* has been studied by means of stereomicroscope equipped with a high definition Leica digital camera, optical microscope (OM), and scanning electron microscope (SEM). Plant material used for SEM microscopic examination was fixed in glutaraldehyde (3% in 0.05 M phosphate buffer), dehydrated in a graded acetone series, and dried in a Balzers Critical Point Drier 020. The samples were mounted on aluminum stubs and sputter-coated with gold for 3 min at 15 mA current. Observations were carried out with a Philips XL30/CP Scanning Electron Microscope at 20 kV.

2.3. Vitality Test

Seeds of *A. mathildae* were subjected to the Triphenyl Tetrazolium Chloride test (TTC test) in order to check their viability. The TTC test is based on the reduction of the tetrazolium salt 2,3,5-triphenyl-tetrazolium chloride (TTC), an organic dye that highlights the respiratory activity. In the presence of a dehydrogenase, TTC turns from colorless to red (formazan) [25]. Data analysis was performed with the software package Microsoft Excel, version 2010.

2.4. Sterilization of Plant Material and Composition of Media Tested

To obtain sterile seedlings for *in vitro* cultures, seeds were placed in 15 ml Falcon tubes filled with water and a few drops of Tween® 20, and then agitated by vortex(fixed speed 2500 rpm) for 10 minutes. A part of the seeds was subjected to cold-wet stratification treatment to simulate chilling conditions under snowpack typical of high altitude mountain areas, while another part of the seeds was pre-treated with a commercial stimulator of germination containing humic extracts (Germinator®, CIFO) [26]. For stratification treatment, some soil collected from natural stations of *A. mathildae* was placed in aluminum containers (drilled on the bottom for drainage) and 90 seeds of *A. mathildae* were dropped over that, then covered with a light dusting of further soil. Next, the containers were wetted with vaporized water, covered with aluminum foil and placed in a refrigerator at 4˚C for 90 days [27]. With regard to the pre-treatment with Germinator®, 60 seeds were left to soak for 24 hours in 15 ml Falcon tubes containing deionized water plus 5 drops of Germinator®. A group of 30 seeds was subjected to both cold-wet stratification (firstly) and pre-treatment e surface sterilized in 0.1% NaOCl for 18 minutes. After a thorough wash, they were placed on Phytatray II vessels (Sigma) containing Murashige and Skoog (MS) basal medium [28], supplemented with 3% sucrose and 0.7% agar, pH 5.7, and placed in a growth chamber at 22˚C. The seeds were placed to germinate part in the light, with neon Osram L30/41 lamp lighting and a photoperiod of 14 hours, and part in the dark; moreover, several concentrations of 6-benzylaminopurine(BAP) and α-naphthalene acetic acid(NAA) were added in some culture media in order to induce cell differentiation, by germination (see **Table 1**). Data analysis was performed with the software package Microsoft Excel, version 2010.

2.5. Callus Induction

In order to protect individuals and minimize structural damage, we harvested plants from natural station in very small quantities (**Figure 2(a)**). The leaves of *A. mathildae* were both cut into 3×3 mm fragments and halved by cutting along the central rib. The gems used for the explants were both apical and lateral buds present in the rosettes of *A. mathildae* (**Figure 2 (b)**). The *callus* induction was evaluated on 150 leaf explants and 50 buds, both harvested from individuals collected in nature, and 200 cotyledons obtained from seeds germinated *in vitro* [29] (**Figure 2(c)**). The sterilization was carried out according to the protocol described above, except for using a lower residence time in 0.1% NaOCl because the tissue, both of the leaf and the bud, is much softer than the integument of the seed. The residence time was of some minutes, mostly 10 minutes, paying attention to prevent that the tissues would turn yellow. The explants, after a thorough wash, were transferred in Phytatray II vessels containing MS with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), BAP and NAA, either alone or in combination, as shown in **Table 2**, and placed in a growth chamber at 22˚C, with neon Osram L30/41 lamp lighting and a photoperiod of 14 hours. The percentage of *callus* formation, namely the number of *callus*-forming explants relative to the total number of explants, was calculated after 4 weeks. Data analysis was performed with the software package Microsoft Excel, version 2010. Results are expressed as mean ± standard error.

Figure 2. (a) *Androsace mathildae* L. plants in a natural station; (b) *Androsace mathildae* L. specimen harvested from a natural station (in particular, the lateral buds are visible); (c) *Androsace mathildae* L. seeds sprouted *in vitro*.

Table 1. Media tested for seed germination and relative results.

Pre-conditioning	MS	½MS	pH	NAA mg·l⁻¹	BAP mg·l⁻¹	2,4-D mg·l⁻¹	Hydrolized Casein mg·l⁻¹	CaCO₃ mg·l⁻¹	Dark	Temperature (°C)	Germination percentage
-	●		5.7								10
-	●		5.8				500	500			0
-	●		5.7						●		0
Stratification	●		5.7						●		15
Stratification	●		5.7								13
Germinator®	●		5.7						●		93.3
GA₃ (gibberellic acid) 3 mg·l⁻¹ × 15 h		●	5.8							4	30
-	●		5.7	0.10	0.5						62.5
-	●		5.7	0.25	0.5						92.5
-	●		5.7	0.25	0.5				●		92.5
Stratification	●		5.7	0.25	0.5				●		97.4
Germinator®	●		5.7	0.25	0.5						92.3
Germinator® and Stratification	●		5.7	0.25	0.5				●		95
Germinator®	●		5.7	0.25	0.5				●		100
-	●		5.8			1			24 h × 30 days	26	0
-	●		5.8			0.5	500	500			0
-	●		5.8			1					0
-		●	5.8		0.5	0.5					0

2.6. Neomorphogenesis

The samples that demonstrated a better growth condition (with respect to mass, hardness and color) were transferred to new growth medium consisting of MS, BAP, NAA. To improve measurements of *callus* growth, we performed a visual evaluation [30]. The growth aspects that we have considered consist in the mass, hardness and colour.

After 1 month the frequency of differentiation in sprouts was calculated, expressed as number of *calli* with buds on the total number of *calli* × 100. Subsequently, the *calli* with the shoots were transferred to a fresh culture medium similar to the previous one, and the frequency of differentiation to shoot was calculated, expressed as number of *calli* with shoot on the total number of *calli* × 100, calculated after other 30 days. Each treatment was repeated 5 times, and each Phytatray II vessels contained about 13 - 20 *calli*. Data analysis was performed with the software package Microsoft Excel, version 2010. Results are expressed as mean ± standard error.

3. Results

3.1. Morphology and Microscopic Characteristics

Sample observation at SEM allowed to highlight several interesting morphological traits of *Androsace mathildae*, such as the calyx tube with five stamens (**Figure 3(a)**), the magnification of one stamen accreted to the corolla tube (**Figure 3(b)**) and numerous pollen grains (**Figure 3(c)**). The pollen grain is oblate-spheroidal, very small (7 - 8 µm × 12 - 14 µm) and tricolpate, with long and narrow colpi, the exine is foveolated (**Figure 3(d)**). The slightly ovoidal seed capsule, once ripened, opens up in five valves (**Figure 3(e)**); generally the capsule contains five seeds (about 1.5 mm × 1 mm) with reticulated seed coat (**Figure 3(f)**). Scanty hairs (about 100 µm long) are
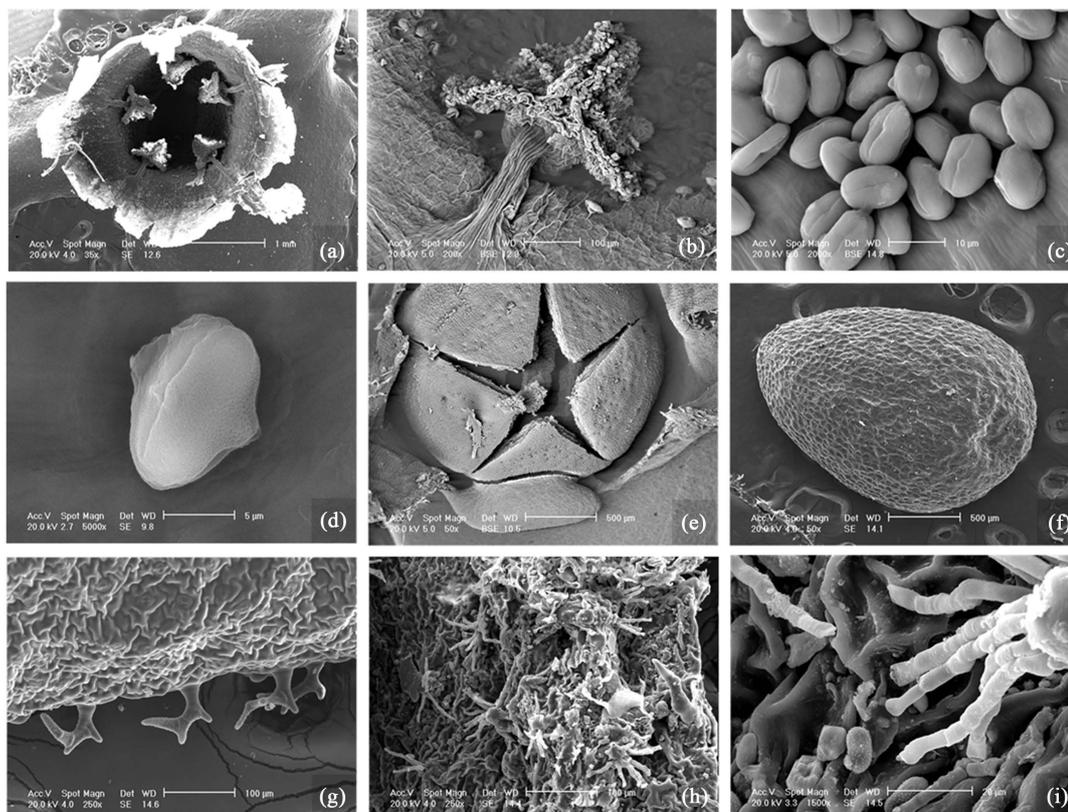
Figure 3. Morphological observations at scanning electron microscopy of *Androsace mathildae* L.: (a) Calyx tube with five stamens (scale bar = 1 mm); (b) Magnification of stamen accreted to the corolla tube (scale bar = 100 µm); (c) Pollen grains (scale bar = 10 µm); (d) Detail of pollen grain in equatorial view (scale bar = 5 µm); (e) Seed capsule (scale bar = 500 µm); (f) Seed coat detail (scale bar = 500 µm); (g) Leaf margin detail (scale bar = 100 µm); (h) Fungal hyphae on the leaves (scale bar = 100 µm); (i) Detail thereof (scale bar = 20 µm).

found on the leaf apex (**Figure 3(g)**); this noteworthy feature allows to distinguish *A. mathildae* from *A. komovensis* sp. nov., which, conversely, exhibits leaves with a clearly ciliated margin [23]. The leaf analysis at higher magnitude pointed out the presence of fungal hyphae not yet identified (**Figure 3(h)** and **Figure 3(i)**); previous studies on *Androsace* genus reported the presence of actinomycetes named *Agromyces albus* sp. nov. over the aerial part of the plant [31].

The stereo and optical microscope analysis led to the identification of the ascomycete *Pyrenophora bromi* as an agent of leaf decomposition (**Figure 4(a)** and **Figure 4(b)**). This is a small genus with thick-walled pseudothecia and bearing dark brown bristles on their upper surface [32] [33]. This ascomycete has never been reported either on *A. mathildae*, or at these altitudes.

3.2. Seed Vitality and Germinability

TTC test showed that all the seeds, both those harvested in summer 2008 and those harvested years after, were vital. In fact, all of them exhibited a bright red staining, sign of mitochondrial respiration. Therefore, we showed that prolonged preservation does not cause vitality reduction. *In vitro* seed germinability was calculated 7 weeks after the implantation. The germinability of seeds placed on auxin- and cytokinin-free media was very low (ranging from 0% to 15%) as reported by other authors [34], while the percentage of sprouted seeds on auxin- and cytokinin-containing media and pre-treated with Germinator® was 91% (**Table 1**).

3.3. Plant Material Achievement

The best results with regard to germination, assessed 7 weeks after seed implantation (**Table 1**), were given by auxin- and cytokinin-containing media, with the same percentage for both Germinator® pre-treated and not pre-treated seeds. The main difference lies in the germination time, which was shorter for stimulator-treated seeds. Seeds placed on media without plant growth regulators, or media with only 2,4-D, exhibited a very low germinability and, in addition, the shoots began to get brown just after the germination, becoming unusable after about 2 weeks. In the present study, seed germination and *callus* formation response was higher in MS supplemented with BAP and NAA. In line with the observation reported in development studies in other genera, both low and high concentrations of 2,4-D failed to stimulate seed germination and protocorm development [35] [36]. Cotyledons used in the following explants were harvested from seeds germinated on media reported in **Table 1** with germination over 92%.

3.4. Callus Induction

None of the tested media, containing the various 2,4-D, 2,4-D + BAP and BAP + NAA concentrations specified above, provided positive results for *callus* induction from leaf explants and apical and lateral buds. On the contrary, positive results were obtained for explants derived from *in vitro*-generated cotyledons placed in culture media containing 2,4-D + BAP and BAP + NAA (**Table 2**). The percentage of *calli* formed in the presence of 2,4-D + BAP is not very high and they did not exhibit a good growth, showing a colour tending to dark brown and often an insubstantial mass. Clearly better results were obtained on medium containing BAP + NAA at var-

(a)   (b)
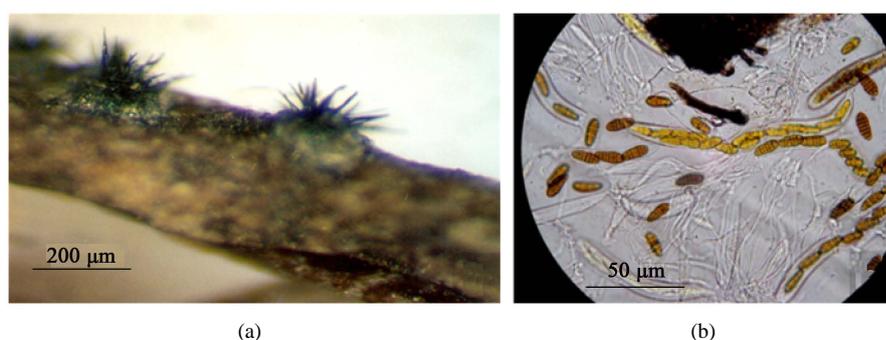
Figure 4. (a) Stereomicroscope analysis showing. *Pyrenophora bromi* with thick-walled pseudothecia and bearing dark brown bristles on decaying leaves (scale bar = 200 μm); (b) Optical microscope analysis showing details of asci and ascospores (scale bar = 50 μm).

Table 2. Media for callus induction tested on cotyledons obtained from *in vitro* germinated seeds. The percentage of callus formation, namely the number of callus-forming explants over the total number of explants × 100, was calculated after 4 weeks.

MS	pH	NAA mg·l⁻¹	BAP mg·l⁻¹	2,4-D mg·l⁻¹	Percentage of *callus*-forming cotyledons ± standard error
x	5.7			0.5	0
x	5.7			0.8	0
x	5.7			1	0
x	5.7		0.1	0.5	0
x	5.7		0.2	0.4	46 ± 13.65
x	5.7	0.10	0.5		92.57 ± 6.47
x	5.7	0.25	0.5		95.02 ± 3.43
x	5.7	0.35	0,5		92.08 ± 9.71
x	5.7	0.25	0.6		91.18 ± 2.44
x	5.7	0.4	0.8		94.77 ± 4.53

ious concentrations (**Table 2**), on which the frequency of *callus* formation is very high, reaching about 93% (**Figure 5**).

The *calli* presented a colour in many cases pale or tending to green, solid mass, excellent growth and strong neomorphogenesis capacity. At the time of observation many of the *calli* were already differentiating, generating the first buds and shoots.

3.5. Neomorphogenesis, Development and Growth of Regenerated Shoots

Transferring on MS medium supplemented with 0.5 mg·l⁻¹ BAP and 0.25 mg·l⁻¹ NAA caused an extremely high frequency (100%) in bud and shoot formation (see **Table 3**).

In particular, the *calli* that were firm and bright green and had a dimension higher than 5 mm showed higher neomorphogenetic capacity (**Figure 6(a)**). The number of buds ranged from 1 to 20 per *callus*, with an average of 7.12, while their dimensions were quite small (about 1 cm) (**Figure 6(b)**). We observed that *calli* remaining in the same medium for prolonged periods (>25 days) were subjected to unwanted phenomena, such as tendency to scorch. It is to be noted that the scorching process, even if diffused on the entire culture, did not cause culture death nor the loss of the neomorphogenetic capacity. In fact, transferring the scorched *calli* on fresh medium allowed the appearance and proliferation of morphogenetic tissue, even several (8 - 12) weeks after the scorch. *A. mathildae* presented a high growth rate but also a high number of lateral buds, which were used to increase the number of clones present in the growth chamber within a few sub-culture passages (**Figure 6(c)**), thus limiting the use of plant growth factors. This part of the study was particularly successful, with 100% of growth of the *shoots*.

4. Discussion

More than half of the vascular plant flora may become endangered by the year 2080 as a result of climatic changes [37]. The Red List evaluations represent the first unfavourable trends in the threat status of plant species [38], as it is in our study case. As specified in Secretariat of the CBD 2002 [39], the success of conservation must be based on a solid knowledge to understand the value of plant diversity. Our study aimed at deepening the morphological analysis of the plant. SEM observation of *Androsace*, performed in our work for the first time, allows us to describe the structural characteristics of a high altitude plant species. The pollen grain is oblate-spheroidal, the exine is foveolated without echine suggesting that pollination might be ambophily by insects and wind. The androceo consists of five stamens, connate with the corolla tube, and as many carpels welded consisted the ovary, which is overcome and unilocular. The tests we carried out with the TTC on the collected seeds indicate that pollination is successful because the seeds are vital showing mitochondrial respiration. The
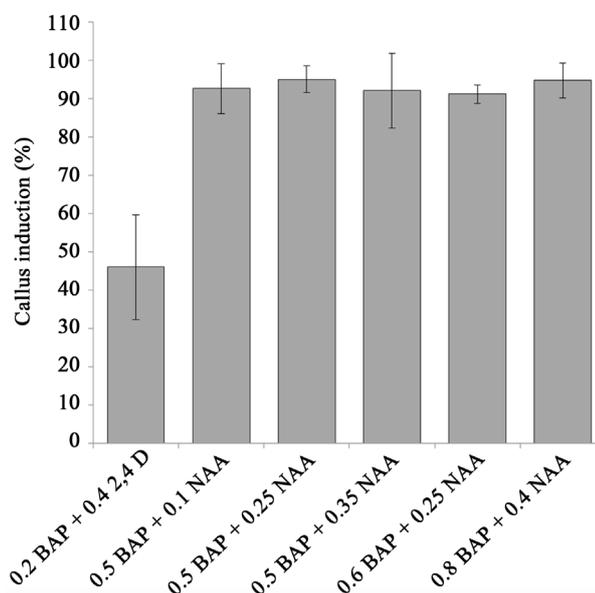
Figure 5. Percentage of *callus* induction from cotyledons in media containing BAP + NAA at various concentrations.
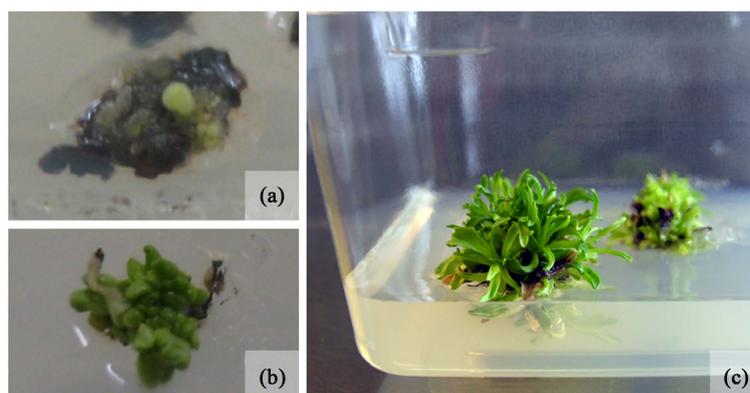
Figure 6. (a) *Androsace mathildae* L. callus with bud primordium; (b) *Androsace mathildae* L. callus with multiple buds; (c) *Androsace mathildae* L. specimen obtained *in vitro*.

Table 3. Media tested for neomorphogenesis induction and relative obtained percentage of buds and shoots formation.

MS	pH	NAA mg·l^{-1}	BAP mg·l^{-1}	Percentage of formed buds and shoots ± standard error
x	5.7		0.1	17.25 ± 2.56
x	5.7		0.3	29.64 ± 0.91
x	5.7	0.10	0.3	57.35 ± 4.69
x	5.7	0.25	0.5	100 ± 0.00
x	5.7	0.25	0.7	79.11 ± 1.80

small number of seeds (about 5) and the size of the seeds, relatively large compared to the plants, are in agreement with previous studies which indicated a trend from larger numbers of small seeds at lower altitude to smaller numbers of larger seeds at higher altitude [40].

The large size of the seed of *A. mathildae* prevents its long-distance diaspore favoring the fall in the vicinity of the basal rosette. We observed that the germination of seeds in the laboratory was increased by the use of ex-

tracts of humic acids. This condition in nature is replicated from the decomposition of the lower leaves of *A. mathildae* which welcome the seeds fallen from the plant's floral mature peduncolate capsule [19]. *Androsace mathildae* can be found in the cracks of limestone at an altitude above 2500 m, with a little substrate, therefore the success of germination is due to the presence of organic material consisting of the same basal rosette decomposition. On the aerial portion of the plant we identified *Pyrenophora bromi*, never reported at these altitudes, as an agent of leaf decomposition. The results obtained by *in vitro* treatments show that the highest percentage (91%) of sprouted seeds was obtained on media with NAA (0.25 mg·l^{-1}) and BAP (0.5 mg·l^{-1}) pre-treated with a solution containing humic extracts. *Callus* induction was obtained from cotyledons of *in vitro* germinated seeds on a medium containing 0.5 mg·l^{-1} 6-benzylaminopurine and0.25 mg·l^{-1} α-naphthalene acetic acid. The frequency of *calli* formation is about 93%, they presented a colour tending to green, solid mass, excellent growth and strong neomorphogenesis response. Development and growth of *shoots* was readily achieved using the same medium at various concentrations. Positive results were obtained in culture media containing 0.5 mg·l^{-1} BAP and 0.25 mg·l^{-1} NAA; we obtained an extremely high frequency (>93%) in *callus*, buds and *shoots* formation.

5. Conclusions

The conservation of endemic species is a primary aim in the activities of a botanical garden. *Ex situ* conservation collections aim at preserving the genetic diversity of target species threatened by climate change and human pressure. Specific conservation action, in order to define an appropriate strategy, includes a thorough knowledge of biological and morphological features of plant species and protocols for *in vitro* preservation finalized to long term conservation of genetic diversity [41]-[43]. The case study presented herein highlights how conventional strategies of propagation, such as the germination of seeds, do not result efficient without the use of germplasm *ex-situ* conservation techniques.

In this regard *in vitro* technologies can be successfully exploited for biodiversity conservation of the Habitats Directive's plants that show a very low seed germination. On the other hand, in high altitude, agamic reproduction represents a strategy widely used by plants [4]. The studies were conducted despite the scant material available due to the small number of individuals present in nature, namely less than 200 [24].

We developed a method for *in vitro* conservation of *A. mathildae*, which proved to be essential to design further methodologies aimed at *ex situ* conservation, such as cryopreservation that allows the storage of plant tissues like shoots [44]. The results demonstrated that an effective *ex situ* preservation strategy of threatened plants is possible through the deepening of the knowledge relating to the single plant studied.

Acknowledgements

Clarification

The proceedings were approved and carried out according to Protocol 207, registered in the Ethics and Animal Use Committee of UENF.

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