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Review

Overview of the Success of In Vitro Culture for Ex Situ Conservation and Sustainable Utilization of Endemic and Subendemic Native Plants of Romania

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Abstract: Romania has a relatively high diversity of plant species, including 3829 vascular and 979 non-vascular spontaneous plant taxa (species and subspecies). Due to uncontrolled harvesting as well as other causes, including climate change and ecological collapse, the speed of species extinction and the narrowing of the genetic base of plant resources has been reported as a critical issue. Therefore, the national Red List of Romanian flora includes 1453 threatened taxa, of which 95 are endemic and 90 subendemic. Many of these have high ornamental, medicinal–cosmetic, and/or aromatic properties. The high extinction risk of these valuable plants has stimulated both the reconsideration of their vital importance as genetic resources and interest in finding effective methods for conservation. Cultivating these phytogenetic resources in a human-controlled environment is of high importance for effective ex situ conservation, which can further serve sustainable exploitation needs and may facilitate in situ conservation actions. In vitro culture is a powerful tool for producing elite plants for cultivation for different purposes. This review summarizes the current knowledge on in vitro multiplication of 22 endemic and subendemic native plants of Romania, examining the materials used, the treatments applied, and the results obtained in each stage of the micropropagation protocol (culture initiation, proliferation, rooting, and acclimatization). The findings from the reviewed studies are presented in a comparative way, and the potential of plant tissue culture in conservation and sustainable exploitation of these Romanian species is outlined.

Keywords: Romanian flora; biodiversity conservation; micropropagation; plant growth regulators; neglected and underutilized plants; phytogenetic resources

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1. Introduction

Biodiversity, representing the primary condition for the existence of human civilization, ensures the life support system and the development of socio-economic systems. Within natural and semi-natural ecosystems, intra- and interspecific connections are established through which material, energetic, and informational exchanges that ensure their productivity, adaptability, and resilience are carried out. These interconnections are extremely complex, as it is difficult to estimate the importance of each species in the functioning of these systems and the potential consequences of their population reduction or extinction, to ensure the long-term survival of ecological systems, the main provider of resources on which human development and well-being depend. In this sense, biodiversity conservation is essential for the survival of all life forms, including humans [1].

There are 1.7 million species of living organisms on Earth, of which more than 400,000 are plants [2]. Europe's high level of geographical and climatic diversity provides diverse habitats, with around 20,000 vascular plants [3]. Romania, in particular, has

a relatively high diversity of plant species, including 3829 vascular and 979 non-vascular spontaneous plant taxa (species and subspecies) [4].

Due to uncontrolled harvesting, inappropriate agricultural and forestry practices, urbanization, pollution, habitat destruction, and ecological fragmentation, as well as causes other than anthropogenic pressure (e.g., climate changes, ecological collapse, competition with non-native invasive species), a large number of the plant resources of the spontaneous flora are in danger of extinction [5,6]. For example, uncontrolled wild collection in the Mediterranean has caused several species to become endangered, such as *Rosmarinus officinalis* L. in Sardinia [7], *Arnica montana* L. and *Gentiana acaulis* L. in Croatia [8], and, in addition, over 30% of Greece's threatened plants suffer from uncontrolled harvesting [9]. Another example is the wild populations of *Sideritis scardica* Griseb., which is locally endemic to the Balkans and is assessed as Near Threatened by the IUCN Global Red List [10]. Reports on wild harvesting of red-listed wild-growing plants from different countries estimate the following percentages of their flora: 20.5% in Bulgaria [11], 43.2% in Hungary [12], 34% in Slovakia [13], 59.2% in the Czech Republic [14], and 29.7% in Italy [15].

According to the Romanian national red lists, 1453 plant taxa are assessed as threatened, of which 95 are endemic and 90 subendemic [16]. Some examples include the Romanian local subendemic *Syringa josikaea* (Hungarian lilac), which is assessed as Endangered by the IUCN Global Red List; the Romanian endemic species *Andryala laevitomentosa*, *Astragalus peterfii*, *Astragalus pseudopurpureus*, *Campanula romanica*, *Dianthus giganteus* subsp. *banaticus*, *Papaver alpinum* subsp. *corona-sancti-stephani*, *Silene nivalis* and the Romanian subendemic species *Dianthus nardiformis*, *Dianthus spiculifolius*, *Dianthus trifasciculatus* subsp. *parviflorus*, *Klasea bulgarica*, *Moehringia jankae*, all of which are assessed nationally as threatened, i.e., Critically Endangered, Endangered, or Vulnerable [16].

Many of the threatened wild-growing plants have high ornamental, medicinal–cosmetic, and/or aromatic properties. Therefore, conservation measures must be applied to prevent the extinction of these valuable plants in the wild. Conservation efforts must focus both on widespread species that, according to the IUCN Red List, are assessed as threatened either locally or globally, and on range-restricted species such as national endemics [3,16]. Biodiversity preservation involves both in situ strategies (protected areas—national and natural parks) and ex situ strategies (arboreta, cultivation in botanical gardens, seed banks, short- and medium-term in vitro conservation, and cryopreservation) [17].

The measure of in situ conservation of threatened and endemic plants is an effective tool for protection against extinction [6]. This method of conservation involves maintaining the species in their original habitat, in the places where the species are found naturally. Through this method, the species in their habitat are conserved and the ecosystem in which they participate is protected. However, in situ conservation can be difficult to achieve due to various limitations such as the adequate size of areas to preserve target-species' genetic diversity, space, high costs, and skilled personnel required, as well as other difficulties that can be cumbersome to manage, and vulnerability to natural vagaries [18].

For threatened and endemic taxa for which in situ conservation actions are lacking, ex situ conservation measures should be taken as a priority [19]. In some cases, ex situ techniques are the only conservation methods possible for certain species [20]. Ex situ conservation involves preserving threatened plant species outside of their original habitat by placing them in a human-controlled environment. Domestication and cultivation of threatened and endemic plants can be difficult when initial propagating material is limited [21,22]. Compared with conventional methods, plant-tissue culture requires a small amount of initial plant material for plant propagation and conservation actions [23,24]. Among the advantages of in vitro culture techniques are its speed (over 1,000,000 plants can be produced from one explant in a year), it ensures the production of pathogen-free plants, and it can take place throughout the year without seasonal dependence, ensuring the production of biological material with genetic and physiological uniformity [25].

Like most countries in the world, Romania has made efforts to protect threatened taxa through both conventional methods and biotechnological programs. More than 50% of

Romania's threatened wild flora is protected by classic in situ conservation strategies, and 642 plant species are preserved by ex situ conservation techniques. Among them, 524 taxa are housed in Romanian botanical gardens, 156 species are stored in seed banks, 64 plant species are conserved by in vitro cultures nationwide (52 short-term and 11 medium-term in vitro cultures), and 152 taxa are found in ex situ collections worldwide [16]. Cryopreservation of threatened plant species in Romania is limited so far, but long-term storage protocols have been developed for eight taxa [16].

To our knowledge, the scientific literature contains few review studies on the use of in vitro culture in the conservation of threatened taxa in Romania [26,27], these not being recent, and no review is available on Romanian endemic and subendemic plants. In this context, the aim of this review was to obtain an overview of the use of biotechnological programs in the conservation initiatives of endemic and subendemic native plants of Romania through a systematic and comprehensive literature search. Several scientific databases were accessed (Web of Science, Scopus, Google Scholar, PubMed, ResearchGate), as well as conference proceedings and books, resulting in a total of 120 studies included in this review. The vascular taxa (species and subspecies) were selected based on their assessment as Rare [R], Near Threatened [NT], Vulnerable [VU], Endangered [EN], Critically Endangered [CR], Extinct in the Wild [EW], or Extinct [EX] according to at least one of the following national, regional, or global red lists: Boșcaiu et al. [28], Dihoru and Dihoru [29], Oltean et al. [30], Dihoru and Negrean [31], Witkowski et al. [32], Bilz et al. [33], Turis et al. [34], and IUCN Red List [3]. After this selection procedure, only range-restricted taxa from Romania, such as endemics and subendemics of Romanian phytogeographical regions, were included in the review.

2. Biotechnological Approaches for Ex Situ Conservation of Red-Listed Endemic and Subendemic Romanian Plants

To supplement in situ conservation methods and conventional ex situ conservation strategies, biotechnological methods such as short-term and medium-term in vitro culture and cryopreservation have been used for some of the threatened taxa in Romania. Even though research on in vitro micropropagation of many plant species from the Red List of the Romanian flora has been carried out as part of research projects, not all results have been published, as in the case of *Silene zawadski* and *Silene dinarica*.

In this study, in vitro conservation protocols for 22 Romanian endemic and subendemic taxa with conservation priority were examined (Table 1).

2.1. Short-Term In Vitro Cultures

For some threatened taxa, conventional propagation methods may be inefficient due to limited initial plant material. In order to produce sufficient biological material for conservation efforts, alternative techniques such as in vitro culture may be necessary. In vitro culture is a rapid, efficient, and reproducible method of propagating conservation priority species, and it only requires a small amount of plant material to initiate cultures. Additionally, micropropagation has the potential to produce many plants in a short period of time throughout the year [35].

The use of biotechnological programs, such as in vitro culture, in threatened plant conservation initiatives is demonstrated by numerous published articles. In Romania, the first attempts to apply in vitro culture for the ex situ conservation of threatened plant species began in the 1990s [36,37]. Currently, in vitro protocols have been developed for 64 taxa (4.3% of red-listed taxa) nationally, while 152 taxa (10.5% of red-listed taxa) are found in ex situ collections worldwide [16].

Table 1. List of red-listed endemic and subendemic plants native to Romania reviewed in this study (listed alphabetically by scientific name) with their extinction risk assessments to date.

Family	Taxon	(sub)End. Ro [16]	Threat Category							
			Boşcaiu et al. [28]	Dihoru and Dihoru [29]	Oltean et al. [30]	Dihoru and Negrean [31]	Witkowski et al. [32]	Bilz et al. [33]	Turis et al. [34]	IUCN Red List [3]
Asteraceae	*Andryala laevitomentosa* (Nyár.) Greuter	End.	EN	-	EN	CR	EN	DD	CR	DD
Fabaceae	*Astragalus peterfii* Jav.	End.	R	EN	EN	CR	EN	DD	-	DD
Fabaceae	*Astragalus pseudopurpureus* Gusul.	End.	R	VU	VU	EN	VU	DD	EN	DD
Campanulaceae	*Campanula romanica* Savul.	End.	-	VU	VU	EN	-	DD	-	DD
Asteraceae	*Centaurea reichenbachii* DC.	subEnd.	-	R	R	-	-	-	-	-
Caryophyllaceae	*Cerastium transsilvanicum* Schur	End.	-	R	R	-	-	-	-	-
Caryophyllaceae	*Dianthus callizonus* Schott & Kotschy	End.	R	R	R	LC	-	-	-	-
Caryophyllaceae	*Dianthus giganteus* d'Urv. subsp. *banaticus* (Heuff.) Tutin	End.	-	VU	R	-	-	-	-	-
Caryophyllaceae	*Dianthus glacialis* Haenke subsp. *gelidus* (Schott, Nyman & Kotschy) Tutin	subEnd.	-	R	R	-	-	-	-	-
Caryophyllaceae	*Dianthus henteri* Heuff. ex Griseb. & Schenk	End.	-	-	LC	-	-	-	VU	-
Caryophyllaceae	*Dianthus nardiformis* Janka	subEnd.	-	VU	VU	VU	-	-	-	-
Caryophyllaceae	*Dianthus spiculifolius* Schur	subEnd.	-	VU	R	-	-	-	-	-
Caryophyllaceae	*Dianthus trifasciculatus* Kit. subsp. *parviflorus* Stoj. & Aht.	subEnd.	-	R	R	CR	-	-	-	-
Asteraceae	*Doronicum carpaticum* (Griseb. & Schenk) Nym.	subEnd.	-	-	R	-	-	-	-	-
Caryophyllaceae	*Gypsophila petraea* (Baumg.) Rchb.	subEnd.	-	-	R	-	-	-	-	-
Asteraceae	*Hieracium pojoritense* Wol.	End.	-	R	R	-	-	-	-	-
Asteraceae	*Klasea bulgarica* (Acht. & Stoj.) J. Holub [syn. *Serratula bulgarica* Achtaroff et Stoj]	subEnd.	-	VU	VU	VU	-	-	-	-
Caryophyllaceae	*Moehringia jankae* Griseb. ex Janka	subEnd.	R	VU	R	VU	-	DD	-	DD
Papaveraceae	*Papaver alpinum* L. subsp. *corona-sancti-stephani* (Zapal.) Borza	End.	VU	R	R	-	-	-	-	-
Asteraceae	*Saussurea porcii* Degen	subEnd.	EX	EX	EX	EX	CR	-	CR	-
Caryophyllaceae	*Silene nivalis* (Kit.) Rohrb. [syn. *Lychnis nivalis* Kit.]	End.	R	VU	VU	VU	VU	-	VU	-
Oleaceae	*Syringa josikaea* J. Jacq. ex Rchb. f.	subEnd.	EN	R	VU	LC	EN	DD	NT	EN

R—Rare; VU—Vulnerable; EN—Endangered; CR—Critically Endangered; EX—Extinct; LC—Least Concern; DD—Data Deficient; NT—near threatened.

Short-term in vitro micropropagation protocols have been reported for several endemic and subendemic taxa. Although each species requires specific protocols, the in vitro propagation technology consists of four main stages: culture initiation, multiplication, rooting, and acclimatization [38].

2.1.1. Initiation of In Vitro Culture

The initiation of culture is the first stage of the micropropagation method. The success of this stage depends on selecting the appropriate type of explant and the optimal physiological development phase of the plant for sampling. The quality of the initial explants has an important role in the success of the conservation procedure. In general, meristematic tissues in early stages of development have faster growth and are therefore more effective, which is directly related to the potency of the cells. Additionally, for good quality, explants should be taken from mother plants that have been selected for their health and varietal authenticity [39].

To initiate in vitro cultures of the studied taxa, shoot tips, nodal segments, or seeds were typically used as explants (Table 2). The initial propagation material (seeds, cuttings, stolons, or whole plants) was collected from mother stock plants conserved in botanical garden collections or from the wild.

The major challenge in initiating tissue culture is overcoming contamination, especially when dealing with a limited amount of genetic material from an endemic plant [40,41]. In general, the selection of the appropriate sterilizing agent depends on its efficiency and impact on the subsequent development of the plant. For the studied taxa, different concentrations and exposure times of mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) were typically used for disinfecting explants, depending on the type of explant and the physiological development phase of the mother plant from which the explants were obtained. However, sterilization with $HgCl_2$ was difficult for *Doronicum carpaticum* explants [42] and had an inhibitory effect on seed germination of *Moehringia jankae* [43]. In several cases, a pretreatment with ethanol (C_2H_6O) was effective for disinfecting explants [26,42–53]. When seeds were used as explants to initiate in vitro cultures of the studied taxa, special treatments were sometimes required to overcome seed dormancy. This was the case for *Centaurea reichenbachii*, *Dianthus glacialis* subsp. *gelidus*, *Dianthus henteri*, and *Dianthus nardiformis*, where the use of 10% hydrogen peroxide (H_2O_2) was required for successful seed germination in vitro [44,54–56].

Basic media with mineral salts MS [57] and vitamins MS or B_5 [58] were typically used to inoculate explants during the initiation phase of in vitro cultures of most endemic and subendemic plants native to Romania with conservation priority. However, Woody Plant medium (WPM) [59] and Schenk and Hildebrandt medium (SH) [60] were only used for tissue culture establishment for the deciduous shrub *Syringa josikaea* [27,61]. Addition of Fe^{2+} in chelated form in the basic medium MS [57] was essential for the viability of *Saussurea porcii* explants [62] (Table 2).

Plant growth regulators (PGRs) play a major role in determining the development pathway of plant cells in tissue culture. The most widely used PGRs in in vitro culture are auxins and cytokinins. The ratio of auxin to cytokinin is essential in establishing the preferred type of culture. In the initiation phase of in vitro cultures of most of the studied taxa, the basic medium was supplemented with cytokinins (2-isopentyl adenine—2iP, 6-benzylaminopurine—BAP, kinetin—Kin) in combination with auxins such as indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA). In some cases, such as *Centaurea reichenbachii* [44], *Dianthus callizonus* [63,64], *Dianthus henteri* [56], and *Klasea bulgarica* [52], a culture medium without PGRs was used to initiate in vitro cultures. The addition of cysteine as an antioxidant is important for obtaining viable explants of *Saussurea porcii* [62]. In the case of *Hieracium pojoritense*, the culture medium supplemented with ascorbic acid determined the formation of shoots through direct organogenesis, using special foliar cuttings and floral buds [46,65], while zeatin or hydrolyzed casein addition had an unfavorable effect on plant regeneration [46] (Table 2).

The success rate of establishing in vitro cultures of Romanian endemic and subendemic plants with conservation priority varied from 10 to 100%. Although lower success rates were obtained in some cases, the explants that began to grow were sufficient as initial biological material for the subsequent stages of micropropagation (Table 2).

2.1.2. In Vitro Multiplication Stage

An important step in establishing germplasm collections in vitro is multiplying shoots. The composition of culture media, particularly mineral salts and plant growth regulators, plays a crucial role in achieving high propagation rates.

For shoot proliferation, basal media with mineral salts MS [57] and vitamins MS or B_5 [58] proved to be the preferred media of endemic and subendemic plants native to Romania with conservation priority. However, only for the deciduous shrub *Syringa josikaea*, Woody Plant medium (WPM) [59] and Schenk and Hildebrandt medium (SH) [60] were used in the multiplication stage [27,61] (Table 2). The WPM contains a third to a quarter of the level of macroelements compared with MS medium and is often used for species that are difficult to propagate in vitro, such as species of the *Fagaceae* family [66].

Different plant growth regulators were used in the studies of endemic and subendemic plants native to Romania with conservation priority. The use of cytokinins (2iP, BAP, Kin) in combination with auxins (IAA, NAA, and IBA) was efficient in most cases, resulting in high multiplication rates, particularly in different *Dianthus* species [48,54,55]. The study of Marchenko et al. [62] revealed that the addition of NAA and Kin during the first passage and an increased amount of auxins combined with 1 mg/L BAP were not suitable for in vitro cultivation of *Saussurea porcii*. To maintain long-term conservation of *Saussurea porcii*, it was important to halve the amount of PGRs (IAA and BAP) after the fifth subculture passage [62]. For *Hieracium pojoritense*, it was not recommended to keep the tissue on the same medium (MS supplemented with BAP, NAA, and ascorbic acid) for more than 21 days, as it caused tissue necrosis and cellular death [46].

Adenine sulfate was used for *Astragalus peterfii* [67] and *Dianthus spiculifolius* [26] when other PGRs were not sufficiently effective. Cristea et al. [68] demonstrated the efficiency of thidiazuron as cytokinin added to the culture medium, resulting in a multiplication rate of 28 generated microplants/inoculum in the case of *Silene nivalis* [68].

Research on plant regeneration by somatic embryogenesis has also been reported for various threatened species [69,70]. In the case of Romanian endemic and subendemic plants with conservation priority, this was reported for *Andryala laevitomentosa* [71,72], *Astragalus pseudopurpureus* [45,65], *Klasea bulgarica* [52], and *Papaver alpinum* subsp. *corona-sancti-stephani* [73]. However, for conservation purposes, indirect regeneration (via callus) is preferably avoided due to the risk of somaclonal variation and alteration of the adaptive capacities of microplants when transferred to their original habitat [74].

Table 2. Overview of the optimal parameters for the initiation and multiplication stages of in vitro culture of Romanian endemic and subendemic plants with conservation priority (listed alphabetically by scientific name).

Taxon	Type of Explant	Disinfection of Explants	Initiation Media (Basic + PGRs)	Culture Initiation Success (%)	Multiplication Media (Basic + PGRs)	Time Interval after Inoculation	Multiplication Rate (No. of Shoots/Explant or %)	Shoot Length (cm)	Reference
Andryala laevitomentosa	petiole	-	MS + 1 mg/L 2,4-D + 1 mg/L Kin	-	MS, Free	-	-	-	[71]
Andryala laevitomentosa	leaves	3% NaOCl for 10–15 min	MS + 2 mg/L BAP + 0.2 mg/L IAA	-	MS, Free	-	-	-	[72]
Astragalus peterfii	seeds	0.1% $HgCl_2$, NaOCl, Domestos, 98° C_2H_6O	MS $\frac{1}{2}$, Free	-	MS + 0.2 mg/L NAA + 0.5 mg/L Kin + 40 mg/L $AdSO_4$	1 month	4–5	5.4	[67]
Astragalus pseudopurpureus	leaves, petiole	70° C_2H_6O for 1 min, 0.1% $HgCl_2$ for 6–7 min	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	MS + Vit B_5 + 2 mg/L BAP + 2 mg/L Kin + 0.3 mg/L NAA	-	7–10	-	[45]
Astragalus pseudopurpureus	leaves	0.1% $HgCl_2$ for 5–10 min	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	MS + Vit B_5 + 1 mg/L 2,4-D + 0.5 mg/L Kin	-	-	-	[65]
Campanula romanica	nodal segments	70% C_2H_6O for 20 s, 0.5% NaDCC for 5 min	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	80	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	40 days	12	-	[53]
Centaurea reichenbachii	seeds	96% C_2H_6O for 40 s, 10% H_2O_2 for 15 min	MS $\frac{1}{2}$ + Vit B_5, Free	18	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	10–15	-	[44]
Cerastium transsilvanicum	single node stem fragments	70° C_2H_6O for 30 s, $HgCl_2$ for 5–6 min	Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 0.1 mg/L BAP + 0.01 mg/L NAA	-	Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 0.1 mg/L BAP + 0.01 mg/L NAA	-	15–20	-	[47]
Cerastium transsilvanicum	young shoots	0.1% $HgCl_2$ for 5–10 min	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	-	-	[65]
Dianthus callizonus	seeds	0.1% $HgCl_2$ for 10 min	MS, Free	80	-	-	-	-	[64]
Dianthus callizonus	single node stem fragments	70° C_2H_6O for 30 s, 0.1% $HgCl_2$ for 5–6 min	-	-	MS + Vit B_5 + 1 mg/L BAP + 1.5 mg/L Kin + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 0.5 mg/L GA_3	2 months	43	-	[48]
Dianthus callizonus	seeds	0.1% $HgCl_2$ for 20 min	MS, Free	-	MS + 1 mg/L BAP + 0.1 mg/L NAA	10 days	6	-	[74]
Dianthus giganteus subsp. *banaticus*	vegetative shoots (stolons) with 2–3 compact internodes	10% Domestos for 5 min, 0.2% $HgCl_2$ for 5 min	MS + 1 mg/L BAP + 1 mg/L NAA	60–92	MS + 1 mg/L BAP + 0.1 mg/L NAA	30 days	6.75–18.2	-	[75]

Table 2. *Cont.*

Taxon	Type of Explant	Disinfection of Explants	Initiation Media (Basic + PGRs)	Culture Initiation Success (%)	Multiplication Media (Basic + PGRs)	Time Interval after Inoculation	Multiplication Rate (No. of Shoots/Explant or %)	Shoot Length (cm)	Reference
Dianthus giganteus subsp. *banaticus*	nodal segments	5% Domestos, 80% C_2H_6O	MS + 1 mg/L BAP + 1 mg/L NAA	-	MS + 10 mg/L 2iP + 0.1 mg/L NAA	110 days	19	-	[76]
Dianthus giganteus subsp. *banaticus*	seeds	2.4% Domestos for 10 min	MS + 1 mg/L BAP + 0.5 mg/L IBA	83.33	MS + 1 mg/L BAP + 0.1 mg/L NAA	30 days	12	-	[50]
						120 days	46.3	8.35	
Dianthus glacialis subsp. *gelidus*	seeds	90–100% Domestos for 15 min or 4% H_2O_2 for 12 h, 99% C_2H_6O for 1 min, 10% H_2O_2 for 18 min	MS + Vit B_5	100	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	56 days	55	-	[54]
Dianthus glacialis subsp. *gelidus*	uninodal fragments	70° C_2H_6O for few seconds, 0.1% $HgCl_2$ for 5–6 min	MS + Vit B_5 + 1 mg/L BAP + 1.5 mg/L Kin + 0.25 mg/L NAA + 0.5 mg/L GA_3	-	MS + Vit B_5 + 1 mg/L BAP + 1.5 mg/L Kin + 0.25 mg/L NAA + 0.5 mg/L GA_3	-	-	-	[42]
Dianthus glacialis subsp. *gelidus*	single node stem fragments	70° C_2H_6O for 30 s, 0.1% $HgCl_2$ for 5–6 min	-	-	MS + Vit B_5 + 1 mg/L BAP + 1.5 mg/L Kin + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 0.5 mg/L GA_3	2 months	43.3	-	[48]
Dianthus henteri	seeds	4% H_2O_2 for 13 h, 96% C_2H_6O for 2 min, 10% H_2O_2 for 19 min	MS + Vit B_5, Free	75	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	44 days	6.9–7.7	2.9	[56]
						83 days	16	3.1	
Dianthus henteri	seeds	2.4% Domestos for 10 min	MS + 1 mg/L BAP + 0.5 mg/L IBA	52.5	MS + 1 mg/L BAP + 0.1 mg/L NAA	30 days	6.7	-	[50]
						120 days	28.4	5.88	
Dianthus nardiformis	seeds	70° C_2H_6O for 30 s, 2.4% Domestos for 10 min or 70° C_2H_6O for 30 s, 2.5% H_2O_2 for 16 h, 10% H_2O_2 for 15 min	MS + Vit B_5 + 1 mg/L BAP + 1 mg/L Kin + 0.2 mg/L 2,4-D	-	MS + Vit B_5 + 1 mg/L BAP + 1 mg/L Kin + 0.2 mg/L 2,4-D	2 months	40–50	-	[55]
Dianthus nardiformis	shoots fragments	70° C_2H_6O for 30 s, 0.1% $HgCl_2$ for 10 min	MS + Vit B_5 + 1 mg/L BAP + 1 mg/L Kin + 0.2 mg/L 2,4-D	-	MS + Vit B_5 + 1 mg/L BAP + 1 mg/L Kin + 0.2 mg/L 2,4-D	40 days	8.3	-	[77]
	seeds	70° C_2H_6O for 30 s, 2.4% Domestos for 10 min or 70° C_2H_6O for 30 s, 2.5% H_2O_2 for 16 h, 10% H_2O_2 for 15 min							

Table 2. *Cont.*

Taxon	Type of Explant	Disinfection of Explants	Initiation Media (Basic + PGRs)	Culture Initiation Success (%)	Multiplication Media (Basic + PGRs)	Time Interval after Inoculation	Multiplication Rate (No. of Shoots/Explant or %)	Shoot Length (cm)	Reference
Dianthus spiculifolius	seeds	-	MS	-	MS + 1 mg/L 2iP + 0.1 mg/L NAA	4 weeks	10.2	-	[78]
Dianthus spiculifolius	apical, uninodal fragments	100% Domestos for 15 min	MS + Vit B_5 + 1 mg/L Kin + 1 mg/L NAA	30	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	28 days	9.3	-	[79]
						45 days	31.8	-	
Dianthus spiculifolius	apices with 2–3 nodes from young shoots	0.2% $HgCl_2$ for 10 min	MS + Vit B_5 + 4.44 µM BAP + 5.37 µM NAA	100	MS + Vit B_5 + 4.44 µM BAP + 0.54 µM NAA	60 days	17.6–199.2	-	[80]
Dianthus spiculifolius	shoot tips	20% Domestos for 10 min	MS + Vit B_5 + 4.44 µM BAP + 5.37 µM NAA	99.3–100	MS + Vit B_5 + 4.44 µM BAP + 0.54 µM NAA	60 days	23.6–49.9	-	[81]
Dianthus spiculifolius	flower buds, single node stem fragments	70° C_2H_6O for 30 s, $HgCl_2$ for 5–6 min	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 1 mg/L + BAP + 0.1 mg/L NAA	-	30	-	[47]
					Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 0.1 mg/L BAP + 0.01 mg/L NAA	-	30	-	
Dianthus spiculifolius	single node stem fragments	70° C_2H_6O for 30 s, 0.1% $HgCl_2$ for 5–6 min	-	-	MS + Vit B_5 + 1 mg/L BAP + 1.5 mg/L Kin + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 0.5 mg/L GA_3	2 months	83.3	-	[48]
Dianthus spiculifolius	knot, apex	3–10% NaOCl for 8–30 min	MS + 2 mg/L IBA + 2 mg/L BAP + 40 mg/L $AdSO_4$	80	MS + 2 mg/L IBA + 2 mg/L BAP + 40 mg/L $AdSO_4$	2 months	90	-	[26]
Dianthus spiculifolius	seeds	2.4% Domestos for 10 min	MS + 1 mg/L BAP + 0.5 mg/L IBA	70.83	MS + 1 mg/L BAP + 0.1 mg/L NAA	30 days	7.8	-	[50]
						120 days	32.6	4.98	
Dianthus trifasciculatus subsp. *parviflorus*	single node stem fragments	70° C_2H_6O for 30 s, 0.1% $HgCl_2$ for 10 min	MS + Vit B_5 + 1 mg/L BAP + 1 mg/L Kin + 0.2 mg/L NAA	-	MS $\frac{1}{2}$ + Vit B_5 + 0.01 mg/L NAA	30 days	13.33	-	[51]
Doronicum carpaticum	leaves, petiole	70^0 C_2H_6O for few seconds, 0.1% $HgCl_2$ for 4–5 min	MS + Vit B_5 + 1 mg/L BAP + 0.25 mg/L NAA + 1.5 mg/L Kin + 0.5 mg/L GA_3	-	MS + Vit B_5 + 1 mg/L BAP + 0.2 mg/L NAA + 1 mg/L Kin + 0.08 g/L Ad	-	2–5	-	[42]
			MS + Vit B_5 + 1 mg/L BAP + 0.2 mg/L NAA + 1 mg/L Kin + 0.08 g/L Ad	-					

Table 2. *Cont.*

Taxon	Type of Explant	Disinfection of Explants	Initiation Media (Basic + PGRs)	Culture Initiation Success (%)	Multiplication Media (Basic + PGRs)	Time Interval after Inoculation	Multiplication Rate (No. of Shoots/Explant or %)	Shoot Length (cm)	Reference
Gypsophila petraea	cotyledon, hypocotyl, root	-	MS + 1 mg/L Kin + 2 mg/L Ad + 0.1, 0.25, 0.5 or 1 mg/L 2,4-D	-	MS + 1 mg/L Kin + 2 mg/L Ad + 0.1 mg/L 2,4-D	-	-	-	[82]
Gypsophila petraea	seeds	70^0 C_2H_6O for 30 s, 0.1% $HgCl_2$ for 10 min	MS + Vit B_5 + 1 mg/L BAP + 0.5 mg/L NAA + 1 mg/L Kin	100	MS + Vit B_5 + 1 mg/L BAP + 0.5 mg/L NAA + 1 mg/L Kin	-	5	-	[49]
Hieracium pojoritense	leaves, petiole, floral bud	70^0 C_2H_6O for 1 min, 0.1% $HgCl_2$ for 5–7 min	MS + Vit B_5 + 15 mg/L ascorbic acid + 1 mg/L BAP + 0.1 mg/L NAA	-	MS + Vit B_5 + 15 mg/L ascorbic acid + 1 mg/L BAP + 0.1 mg/L NAA	21 days	-	-	[46]
Hieracium pojoritense	foliar cuttings	0.1% $HgCl_2$ for 5–10 min	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	25 days	20–40	-	[65]
Klasea bulgarica	seeds	70% C_2H_6O for 40 s, 0.5% NaDCC for 5 min	MS	87	MS + 2.5 mg/L 2,4 D + 0.5 mg/L Kin	30 days	-	-	[52]
					MS + 1 mg/L NAA + 0.1 mg/L BAP	40 days	-	-	
					MS + 0.1 mg/L NAA + 1 mg/L BAP + 2 mg/L GA_3	50 days	-	-	
Moehringia jankae	seeds	70% C_2H_6O for 30 s, 2.7% NaDCC for 10 min + 2–3 drops of Tween 20	MS + 0.5 g/L active charcoal + 5 mg/L GA_3	22	MS + Vit B_5 + 0.22 µM TDZ + 0.49 µM IBA	2 months	13	4.65	[43]
					MS + Vit B_5 + 4.4 µM BAP + 0.49 µM IBA	2 months	14	4.95	
					MS + Vit B_5 + 4.5 µM ZEA + 0.49 µM IBA	2 months	13	3.93	
Papaver alpinum subsp. *corona-sancti-stephani*	seeds	0.1% $HgCl_2$ for 10 min	MS	10	-	-	-	-	[64]
Papaver alpinum subsp. *corona-sancti-stephani*	seeds	70% C_2H_6O for 30 s, 0.01% $HgCl_2$ for 10 min	MS + Vit B_5	-	MS + Vit B_5 + 1 mg/L BAP + 1 mg/L Kin + 0.2 mg/L NAA	3 months	35	-	[73]

Table 2. *Cont.*

Taxon	Type of Explant	Disinfection of Explants	Initiation Media (Basic + PGRs)	Culture Initiation Success (%)	Multiplication Media (Basic + PGRs)	Time Interval after Inoculation	Multiplication Rate (No. of Shoots/Explant or %)	Shoot Length (cm)	Reference
Saussurea porcii	seeds	96% C_2H_6O + Tween-80 (2 ± 0.1 min) + NaOCl enriched with active chlorine, "Bilyzna" (TUU6-05743160.001-93) in the ratio 1 × 4 (15 ± 0.1 min)	MS + Fe^{2+} in chelated form + 60 mg/L cysteine + 0.1 mg/L IAA + 1 mg/L BAP	-	MS + Fe^{2+} in chelated form + 60 mg/L cysteine + 0.1 mg/L IAA + 1 mg/L BAP (at the first stage of in vitro culture)	-	-	-	[62]
					MS + Fe^{2+} in chelated form + 60 mg/L cysteine + 0.05 mg/L IAA + 0.5 mg/L BAP (after the fifth subculture passage)	24–28 days	-	-	
Silene nivalis	seeds	$HgCl_2$, H_2O_2, NaOCl	-	60	0.5 or 1 mg/L BAP	62 days	30	-	[68]
					0.5 or 1 mg/L TDZ	62 days	28	-	
Syringa josikaea	apical meristems	-	WPM + 0.25 mg/L IAA + 0.1 mg/L NAA	80	WPM + 0.1 mg/L NAA + 7.5 mg/L BAP	-	-	2	[61]
Syringa josikaea	-	-	MS + 1 mg/L BAP + 0.1 mg/L NAA	-	MS + 1 mg/L BAP + 0.1 mg/L NAA or MS + 0.1 mg/L 2iP + 0.1 mg/L IBA	8 weeks	1.66	4.46 / 7.4	[83]
			MS + 0.1 mg/L 2iP + 0.1 mg/L IBA	-					
			MS + 2 mg/L 2iP + 1 mg/L IBA	-					
Syringa josikaea	knot, apex	NaOCl	MS + 1 mg/L IBA + 0.1 mg/L BAP	70	MS + 1 mg/L IBA + 0.1 mg/L BAP	2 months	3	-	[26]
Syringa josikaea	seeds	-	MS $\frac{1}{2}$ + 3 g/L Cv	-	MS $\frac{1}{2}$ + 3 g/L Cv	-	54%	-	[27]
	knot, apex	-	SH + 170 mg/L KH_2PO_4 + 0.1 mg/L IBA + 0.1 mg/L NAA	-	SH + 170 mg/L KH_2PO_4 + 0.1 mg/L IBA + 0.1 mg/L NAA	-	54%	-	

C_2H_6O—ethanol; $HgCl_2$—mercuric chloride; NaOCl—sodium hypochlorite; NaDCC—sodium dichloroisocyanurate; H_2O_2—hydrogen peroxide; Macro—macroelements; Micro—microelements; Vit—vitamins; MS—Murashige and Skoog medium [56]; SH—Schenk and Hildebrandt medium [59]; Vit B_5—Gamborg vitamins [57]; WPM—Woody Plant medium [58]; 2iP—2-isopentyl adenine; BAP—6-benzylaminopurine; IAA—indole-3-acetic acid; NAA—α-naphthaleneacetic acid; IBA—indole-3-butyric acid; Kin—kinetin; $AdSO_4$—adenine sulfate; GA_3—gibberellic acid; Cv—vegetal coal; TDZ—thidiazuron; Ad—adenin; 2,4-D—2,4-dichlorophenoxyacetic acid; ZEA—zeatin.

2.1.3. In Vitro Rooting Stage

For efficient in vitro propagation protocols, it is essential that the shoots root in large proportions and the regenerated plantlets acclimatize successfully. Rooting is a difficult process in recalcitrant species, and, without roots, the survival rate of acclimatized plants is reduced.

For most taxa of conservation priority reviewed in this research, basal media with mineral salts MS [57] and vitamins MS or B_5 [58] were typically used at the in vitro rooting stage, sometimes with macronutrients or micronutrients reduced by half. Only for the deciduous shrub *Syringa josikaea*, Woody Plant medium (WPM) [59] and Schenk and Hildebrandt medium (SH) [60] were used [27,61].

For microshoot rooting, the culture medium was usually supplemented with auxins alone or in combination with cytokinins. For the species *Dianthus spiculifolius*, some studies reported that PGRs were not necessary for the induction of rhizogenesis [80,81]. In the case of *Centaurea reichenbachii*, the culture medium was supplemented with activated charcoal to improve rooting [44]. In most of the species in which the rhizogenesis process was evaluated, low percentages of rooting were obtained (Table 3).

Table 3. Overview of optimal basic media and plant growth regulators (PGRs) for shoot rooting and acclimatization of in vitro regenerated plants, as reported in in vitro studies on endemic and subendemic plants native to Romania with conservation priority (listed alphabetically by scientific name).

Taxon	Rooting Media (Basic + PGRs)	Rooting Rate (%)	No. Roots/Shoot	Acclimatization Rate (%)	Reference
Andryala laevitomentosa	-	-	-	-	[71]
Andryala laevitomentosa	-	-	-	-	[72]
Astragalus peterfii	MS + 0.2 mg/L NAA + 0.5 mg/L Kin + 40 mg/L $AdSO_4$	36.6	-	-	[67]
Astragalus pseudopurpureus	Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 0.25 mg/L Kin + 1 mg/L IBA + 1 mg/L 2,4-D	-	-	-	[45]
Astragalus pseudopurpureus	MS + Vit B_5 + 1 mg/L 2,4-D + 0.5 mg/L Kin	-	6–12	-	[65]
Campanula romanica	-	-	-	-	[53]
Centaurea reichenbachii	MS + Vit B_5 + 3 g/L active coal	-	-	-	[44]
Cerastium transsilvanicum	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	-	-	[47]
Cerastium transsilvanicum	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	-	-	[65]
Dianthus callizonus	-	-	-	-	[64]
Dianthus callizonus	-	-	-	-	[48]
Dianthus callizonus	MS + 1 mg/L BAP + 0.1 mg/L NAA	-	-	-	[74]
Dianthus giganteus subsp. *banaticus*	-	-	-	-	[75]
Dianthus giganteus subsp. *banaticus*	MS + 0.5 mg/L Kin + 0.1 mg/L NAA	-	23.27	-	[76]
Dianthus giganteus subsp. *banaticus*	MS + 1 mg/L BAP + 0.5 mg/L NAA	40	-	-	[50]
Dianthus glacialis subsp. *gelidus*	MS + Vit B_5 + 1 mg/L Kin + 1 mg/L NAA	-	-	-	[54]
Dianthus glacialis subsp. *gelidus*	-	-	-	-	[42]
Dianthus glacialis subsp. *gelidus*	-	-	-	-	[48]
Dianthus henteri	MS + Vit B_5 + 0.1 mg/L BAP + 1 mg/L NAA	-	8.6	-	[56]

Table 3. *Cont.*

Taxon	Rooting Media (Basic + PGRs)	Rooting Rate (%)	No. Roots/Shoot	Acclimatization Rate (%)	Reference
Dianthus henteri	MS + 1 mg/L BAP + 0.5 mg/L NAA	20	-	-	[50]
Dianthus nardiformis	-	-	-	-	[55]
Dianthus nardiformis	MS $\frac{1}{2}$ + 0.01 mg/L NAA	-	7.17	-	[77]
Dianthus spiculifolius	MS + 1 mg/L 2iP + 1 mg/L NAA	-	9.2	50–60	[78]
Dianthus spiculifolius	MS + Vit B_5 + 1 mg/L Kin + 1 mg/L NAA	-	-	60–80	[79]
Dianthus spiculifolius	MS + Vit B_5	-	5.3–10.1	50–70	[80]
Dianthus spiculifolius	MS + Vit B_5	61.1–86.1	5.1–11.1	-	[81]
Dianthus spiculifolius	Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L NAA	-	-	-	[47]
	Macro MS $\frac{1}{2}$ + Micro MS + Vit B_5 + 0.1 mg/L BAP + 0.01 mg/L NAA	-	-	-	
Dianthus spiculifolius	-	-	-	-	[48]
Dianthus spiculifolius	MS + 2 mg/L IBA + 2 mg/L BAP + 40 mg/L $AdSO_4$	-	40	80	[26]
Dianthus spiculifolius	MS + 1 mg/L BAP + 0.5 mg/L NAA	30	-	-	[50]
Dianthus trifasciculatus subsp. *parviflorus*	MS $\frac{1}{2}$ + Vit B_5 + 0.01 mg/L NAA	-	9.33	80	[51]
Doronicum carpaticum	-	-	-	-	[42]
Gypsophila petraea	-	-	-	-	[82]
Gypsophila petraea	MS + Vit B_5	-	-	-	[49]
Hieracium pojoritense	MS + 0.1 mg/L NAA + 1 mg/L GA_3	-	-	-	[46]
Hieracium pojoritense	-	-	-	-	[65]
Klasea bulgarica	MS + 0.1 mg/L IAA	-	-	-	[52]

Table 3. *Cont.*

Taxon	Rooting Media (Basic + PGRs)	Rooting Rate (%)	No. Roots/Shoot	Acclimatization Rate (%)	Reference
Moehringia jankae	MS + Vit B_5 + 0.22 µM TDZ + 0.49 µM IBA	100	-	50	[43]
	MS + Vit B_5 + 4.4 µM BAP + 0.49 µM IBA	100	-	50	
	MS + Vit B_5 + 4.5 µM ZEA + 0.49 µM IBA	100	-	50	
Papaver alpinum subsp. *corona-sancti-stephani*	-	-		-	[64]
Papaver alpinum subsp. *corona-sancti-stephani*	MS + Vit B_5 + 1 mg/L BAP + 0.1 mg/L Kin + 0.2 mg/L NAA	-	-	-	[73]
Saussurea porcii	Macro MS $\frac{1}{2}$ + Micro MS $\frac{1}{2}$ + Vit MS + 0.1 mg/L IAA + 0.01 mg/L BAP	-	-	80	[62]
Silene nivalis	-	-	-	-	[68]
Syringa josikaea	WPM + NAA	48.4	-	-	[61]
Syringa josikaea	MS + 2 mg/L 2iP + 0.1 mg/L IBA	-	1–2	-	[83]
Syringa josikaea	MS + 1 mg/L IBA + 0.1 mg/L BAP	-	3–4	50	[26]
Syringa josikaea	MS $\frac{1}{2}$ + 3 g/L Cv	-	-	50	[27]
	SH + 170 mg/L KH_2PO_4 + 0.1 mg/L IBA + 0.1 mg/L NAA	-	-	50	

MS—Murashige and Skoog medium [56]; SH—Schenk and Hildebrandt medium [59]; Vit B_5—Gamborg vitamins [57]; WPM—Woody Plant medium [58]; Macro—macroelements; Micro—microelements; Vit—vitamins; NAA—α-naphthaleneacetic acid; Kin—kinetin; $AdSO_4$—adenine sulfate; IBA—indole-3-butyric acid; 2,4-D—2,4-dichlorophenoxyacetic acid; BAP—6-benzylaminopurine; IAA—indole-3-acetic acid; 2iP—2-isopentyl adenine; Cv—vegetal coal; ZEA—zeatin; GA_3—gibberellic acid.

2.1.4. Acclimatization Stage

For an in vitro propagation protocol to be widely applicable, it should produce thousands of plants in a short period of time [84,85]. The efficiency of in vitro propagation protocols is highly dependent on the ability of in vitro regenerated plants to acclimatize. In the reviewed studies, only a few taxa were evaluated in this regard. The acclimatization of endemic and subendemic plants native to Romania with conservation priority depended on how the plants responded in the previous stages of in vitro culture. In some cases, a photoautotrophic period in vitro before being transferred ex vitro favors the acclimatization of microplants [54,79]. The hermetic closure of the culture vessels to prevent infection of the cultures inevitably leads to the consumption of all CO_2 from the atmosphere inside the vessels within the first hours after their closure. In vitro photoautotrophic cultures are generally performed using a solid medium without sucrose by obturating the culture flasks with special filters that allow gas exchange between the culture vessels and the surrounding environment [86]. Under these conditions, in vitro plants that are normally entirely or predominantly heterotrophic can perform photoautotrophic growth in vitro, but less so at the time of ex vitro acclimatization [86].

A success rate of more than 50% acclimatization was considered efficient for the development of in vitro propagation protocols for conservation initiatives of these threatened taxa (Table 3).

During the ex vitro acclimatization of *Moehringia jankae*, only 50% of regenerated plants survived when a semisolid combination of perlite and half-strength MS liquid medium was used [43]. For *Saussurea porcii*, the regenerated plants were transferred to sterile perlite, and were watered weekly with a MS $\frac{1}{2}$ solution. Initially, the regenerated plants were covered with a polyethylene cap to prevent dehydration and wilting [62].

The lower survival rates recorded in some taxa were probably due to limited in vitro rooting.

As can be seen from the above data, in Romania, the genus *Dianthus* has received special attention for ex situ conservation by short-term in vitro cultures. *Dianthus* has a large number of Romanian endemic (5) or subendemic (5) species, representing approximately 23% of the total wild taxa of this genus (44 species and subspecies) [16]. Of all the species reviewed in this study, *Dianthus* has the highest number of red-listed (sub)endemic taxa (7). The most studies on conservation by in vitro culture were carried out on local *Dianthus* species from the Carpathian Mountains, including stenoendemics (*D. callizonus*, Piatra Craiului Massif) [63], Romanian endemics (*D. henteri* and *D. giganteus* subsp. *banaticus*) [56,76], subendemics (*D. glacialis* subsp. *gelidus*, *D. spiculifolius*, and *D. trifasciculatus* subsp. *parviflorus*) [51,87] or regional taxa with Carpatho-Balkan distribution (*D. nardiformis*) [55].

Cultivating these phytogenetic resources in a human-controlled environment is an effective ex situ conservation method that can further serve the needs of sustainable exploitation and may facilitate in situ conservation strategies and actions. The high-value plant material regenerated by in vitro culture can be used in conservation or sustainable exploitation cultivation programs but can also be used to design conservation actions aimed at restoring declining plant populations in their original habitats or introducing neopopulations in habitats from which they have disappeared.

2.2. Medium-Term In Vitro Cultures

The short-term in vitro preservation method is costly as it requires frequent subculturing of the biological material on fresh medium every few weeks [24]. An alternative method for in vitro conservation of plant germplasm that reduces subcultivation frequencies (months) and costs is slow-growth storage. This method mainly involves changes in physical and chemical parameters that lead to reduced growth [88]. Another medium-term storage method is incorporating various dehydrated propagules into synthetic seeds [89].

Since different species have varying responses to the application of growth limiting factors, it is necessary to develop optimized protocols for medium-term in vitro conservation for each species. Worldwide, medium-term in vitro culture studies for threatened and endemic plant species are limited [87,90,91].

To the best of our knowledge, no such protocols have been reported internationally for the taxa reviewed in this study. In Romania, medium-term in vitro conservation protocols have been developed for eight red-listed endemic and subendemic taxa: *Dianthus callizonus*, *D. glacialis* subsp. *gelidus*, *D. spiculifolius*, *D. nardiformis*, *D. trifasciculatus* subsp. *parviflorus*, *Gypsophila petraea*, *Moehringia jankae*, and *Papaver alpinum* subsp. *corona-sancti-stephani* [16].

The best results for growth retardation in in vitro cultures were obtained using mannitol and polyethylene glycol in several *Dianthus* taxa [48,92–96], or flurprimidol, mannitol, and chlormerquat in the case of *Moehringia jankae* [97]. Other strategies for slow growth involved low temperatures (10 °C) and nutrient reduction, which have been proven to be effective for various threatened species such as *Gypsophila petraea* and *Dianthus callizonus* [63]. Catană and Holobiuc [98] demonstrated the efficiency of somatic embryos storage at low temperatures (4 °C and −20 °C) as an ex situ conservation method for *Papaver alpinum* subsp. *corona-sancti-stephani*, but it is worth mentioning that the somatic embryos lost their viability at an ultralow temperature (−75 °C) [98] (Table 4).

2.3. Cryopreservation

Over 36% of threatened species produce recalcitrant seeds that cannot be preserved in seed banks [99]. Additionally, maintaining several species in active culture for an extended period can be difficult [100]. Therefore, cryopreservation (storage in liquid nitrogen at −196 °C) is an efficient method of long-term ex situ conservation of threatened taxa that cannot be preserved by conventional methods and serves as an alternative to short- and medium-term in vitro conservation [101].

Developing effective cryopreservation protocols is essential for species that are recalcitrant to other conservation methods [102]. When developing these protocols, it is important to consider the type of plant (woody or herbaceous) and the predominant method of multiplication (vegetative or seed propagation). Factors that can influence the success of cryopreservation are the type of explant, cryopreservation techniques, pretreatment conditions, and regrowth treatment [102]. The key to successful cryopreservation is maintaining the viability and regenerative capacity of the preserved plant material after it is restored to optimal culture conditions [103].

Worldwide, most studies on plant cryopreservation have focused on crop species and less on threatened or endemic plants [24]. The most widely used methods for cryopreservation of various endemic and threatened plant species were droplet-vitrification [104], vitrification [105,106], encapsulation-vitrification [107], and encapsulation-dehydration [108]. Different types of explants have been used for cryostorage, such as: shoot tips [109], nodal explants [110], in vitro grown buds [111], callus [104], protocorms [112], seeds [113,114], and pollen [115].

For the endemic and subendemic plant species included in this study, cryopreservation protocols have currently been developed for only six *Dianthus* taxa, namely *Dianthus callizonus, D. glacialis* subsp. *gelidus*, *D. henteri*, *D. nardiformis*, *D. spiculifolius*, and *D. trifasciculatus* subsp. *parviflorus*. The explants used for cryostorage in liquid nitrogen by the droplet-vitrification technique were shoot tips and axillary buds from plants micropropagated for two years [116] (Table 5).

While several endemic and threatened plant species worldwide have been successfully cryopreserved with over 50% survival or regrowth rates [105,108], cryopreservation protocols developed at the national level have led to higher survival and regeneration rates in the targeted species (a regeneration rate of 73.3% for the subendemic taxon *D. nardiformis* and a survival rate of 83.3% for the endemic taxon *D. henteri*) [116]. This could be explained by the different types of explants used, pretreatment conditions, cryopreservation procedures, and post-thaw treatment applied [102].

Regarding the number of shoots per explant after cryostorage, it varied between 3 for *D. glacialis* subsp. *gelidus* and *D. henteri*, and 5.6 for *D. nardiformis* [116] (Table 5).

Table 4. Overview of growth inhibitory factors used to induce minimal cultures, as reported in in vitro studies on endemic and subendemic plants native to Romania with conservation priority (listed alphabetically by scientific name).

Taxon	Basic Media + PGRs	Growth Inhibitory Factors	Time Intervals	Regeneration Rate (No. of Shoots/Explant)	Shoot Length (cm)	Viable Shoots (%)	Rooting Rate (%)	Survival Rate (%)	Reference
Dianthus callizonus	MS + Vit B_5, free	3% mannitol	3 months	26.60	-	-	-	-	[48]
Dianthus callizonus	MS1/10 + Vit B_5, free	reduced mineral concentration	12 months	-	-	100	100	-	[63]
	MS + Vit B_5, free	reduced temperature (10 °C)	12 months	-	-	100	-	-	
Dianthus glacialis subsp. *gelidus*	MS + Vit B_5, free	3% mannitol	3 months	14.30	-	-	-	-	[48]
Dianthus glacialis subsp. *gelidus*	MS + Vit B_5, free	0.32 M mannitol	1 months	2–5	0.5	-	-	-	[92]
			2 months	5–10	0.5	-	-	-	
		0.49 M mannitol	1 months	2–4	< 0.5	-	-	-	
			2 months	10–15	<0.5	-	-	-	
Dianthus glacialis subsp. *gelidus*	MS + Vit B_5, free	0.16 M mannitol	3 months	15–20	1	-	-	-	[94]
			6 months	20	1	-	-	-	
Dianthus nardiformis	MS + Vit B_5, free	329 mM mannitol	40 days	2.60	1.80	-	-	-	[96]
			80 days	4.86	2.80	-	-	-	
			120 days	22.26	-	-	-	-	
Dianthus spiculifolius	MS + Vit B_5, free	3% mannitol	3 months	25.00	-	-	-	-	[48]
Dianthus spiculifolius	MS + Vit B_5, free	0.32 M mannitol	1 months	3–5	0.5	-	-	-	[92]
			2 months	5–10	0.5–1	-	-	-	
		0.49 M mannitol	1 months	1–2	<0.5	-	-	-	
			2 months	7–10	<0.5	-	-	-	
Dianthus spiculifolius	MS + Vit B_5, free	0.16 M mannitol	3 months	20–30	1–1.5	-	-	-	[94]
			6 months	-	1.8–2	-	-	-	
Dianthus spiculifolius	MS + Vit B_5, free	0.16 M mannitol	6 months	-	-	-	-	-	[93]
Dianthus trifasciculatus subsp. *parviflorus*	MS + Vit B_5, free	6% PEG 4000	40 days	2.87	3.40	-	-	-	[95]
			80 days	13.40	3.93	-	-	-	
			120 days	35.13	6.00	-	-	-	

Table 4. *Cont.*

Taxon	Basic Media + PGRs	Growth Inhibitory Factors	Time Intervals	Regeneration Rate (No. of Shoots/Explant)	Shoot Length (cm)	Viable Shoots (%)	Rooting Rate (%)	Survival Rate (%)	Reference
Dianthus trifasciculatus subsp. *parviflorus*	MS + Vit B_5, free	3% mannitol	40 days	5.67	3.60	-	-	-	[95]
			80 days	9.93	3.87	-	-	-	
			120 days	47.13	4.67	-	-	-	
Gypsophila petraea	MS1/4 + Vit B_5, free	reduced mineral concentration	12 months	-	-	100	100	-	[63]
	MS + Vit B_5, free	reduced temperature (10 °C)	12 months	-	-	100	45	-	
Moehringia jankae	MS, free	32 µM flurprymidol	1 month	1.36	0.56	-	0	-	[97]
			2 months	2.52	0.75	-	36	-	
			3 months	4.24	0.98	-	76	-	
			6 months	4.60	0.55	-	-	88	
		48 µM flurprymidol	1 month	1.24	0.36	-	0	-	
			2 months	1.60	0.65	-	0	-	
			3 months	2.40	0.81	-	0	-	
			6 months	7.08	0.32	-	-	88	
		0.16 M mannitol	1 month	1.80	0.42	-	32	-	
			2 months	3.88	1.06	-	69	-	
			3 months	5.68	1.11	-	81	-	
			6 months	5.64	1.17	-	-	88	
		2.5 mM clormerquat	1 month	1.72	1.62	-	0	-	
			2 months	3.84	2.37	-	28	-	
			3 months	5.68	2.33	-	72	-	
			6 months	6.68	2.06	-	-	96	
Papaver alpinum subsp. *corona-sancti-stephani*	MS + 3% sucrose + 3% mannitol, free	reduced temperature (4 °C and −20 °C)	3 weeks	-	-	-	-	> 65	[98]

MS—Murashige and Skoog medium [56]; Vit B_5—Gamborg vitamins [57]; PEG—polyethylene glycol.

Table 5. Effect of cryopreservation (droplet-vitrification technique) on shoot survival (rate assessed four weeks after cryopreservation), shoot regrowth (rate assessed six weeks after rewarming), and number of regenerated shoots per explant (assessed after 60 days), as reported in in vitro studies on endemic and subendemic plants native to Romania with conservation priority.

Taxon	Explants Used for Cryostorage	Osmoprotection Treatment	Dehydration Treatment	Cooling Treatment	Regrowth Treatment	Shoot Survival Following Cryostorage (%)	Shoot Regrowth Following Cryostorage (%)	No. of Shoots/Explant Following Cryostorage	Reference
Dianthus callizonus	axillary buds from micropropagated plants for two years	MS medium + 0.25 M sucrose, 24 h at 23 ± 1 °C	PVS2, 30 min at 23 ± 1 °C	liquid nitrogen (−196 °C), 24 h	MS medium + 30 g/L sucrose, 23 ± 1 °C	71.6	65	4.6	[116]
Dianthus glacialis subsp. *gelidus*	shoot tips from micropropagated plants for two years	MS medium + 0.25 M sucrose, 24 h at 23 ± 1 °C	PVS2, 30 min at 23 ± 1 °C	liquid nitrogen (−196 °C), 24 h	MS medium + 30 g/L sucrose, 23 ± 1 °C	70	63.3	3	[116]
Dianthus henteri	shoot tips from micropropagated plants for two years	MS medium + 0.25 M sucrose, 24 h at 23 ± 1 °C	PVS2, 30 min at 23 ± 1 °C	liquid nitrogen (−196 °C), 24 h	MS medium + 30 g/L sucrose, 23 ± 1 °C	83.3	71.6	3	[116]
Dianthus nardiformis	shoot tips from micropropagated plants for two years	MS medium + 0.25 M sucrose, 24 h at 23 ± 1 °C	PVS2, 30 min at 23 ± 1 °C	liquid nitrogen (−196 °C), 24 h	MS medium + 30 g/L sucrose, 23 ± 1 °C	73.3	73.3	5.6	[116]
Dianthus spiculifolius	shoot tips from micropropagated plants for two years	MS medium + 0.25 M sucrose, 24 h at 23 ± 1 °C	PVS2, 30 min at 23 ± 1 °C	liquid nitrogen (−196 °C), 24 h	MS medium + 30 g/L sucrose, 23 ± 1 °C	68.3	66.6	3.6	[116]
Dianthus trifasciculatus subsp. *parviflorus*	shoot tips from micropropagated plants for two years	MS medium + 0.25 M sucrose, 24 h at 23 ± 1 °C	PVS2, 30 min at 23 ± 1 °C	liquid nitrogen (−196 °C), 24 h	MS medium + 30 g/L sucrose, 23 ± 1 °C	71.6	70	5	[116]

MS—Murashige and Skoog medium [56]; PVS2—plant vitrification solution 2 [117].

3. Conclusions and Perspectives

The findings from the reviewed studies showed that in vitro culture techniques are effective not only for increasing the number of regenerated plants in cases where other methods are inadequate, but also for the ex situ conservation of Romanian native species with conservation priority. Although the results for some endemic and subendemic species native to Romania reported in this review are sequential, they represent important data that can be used by further studies aimed at developing optimized in vitro propagation protocols for these valuable species.

For the successful in vitro conservation of endemic plant species, future strategies must include:

- Improvement of short-term in vitro propagation protocols, respective of each stage of micropropagation (initiation, multiplication, rooting, acclimatization), medium- and long-term conservation protocols developed so far. For conservation purposes, it is important to have optimized micropropagation protocols that result in a satisfactory regeneration rate, vigorous and rooted plants, and high viability of acclimatized plants, particularly when dealing with threatened endemic plants whose germplasm is very limited.
- Development of a national database with in vitro protocols elaborated so far (published and unpublished) that can be easily accessed by scientists. For some endemic species, such as *Silene zawadski* and *Silene dinarica*, the in vitro protocols established within some research projects have not yet been published, access to these data being difficult.
- Extension of research on conservation by biotechnological methods to other taxa with conservation priority that have not yet been studied. Among the 1453 threatened Romanian taxa, of which 95 are endemic and 90 subendemic, only 64 are conserved by in vitro cultures nationwide and cryopreservation protocols have been developed for only 8 taxa.
- Medium- and long-term conservation of endemic species within the national germplasm bank. Currently, a small number of species are conserved only within some research institutes.
- Organization of a researchers' network from different fields: botany, genetics, ecology, micropropagation, conservation, etc., for the effective dissemination of information regarding in vitro conservation protocols. In this way, sustainable conservation can be achieved, especially for species that are threatened with extinction.
- Allocation of funds for the development of a national database, gene banks, research programs, etc.
- Elaboration of cryopreservation protocols for all endemic and subendemic plant species. Presently, there are known protocols only for some endemic species of the *Dianthus* genus [116].
- Genetic diversity evaluation of the endemic and subendemic species using techniques such as AFLP, RAPD, RFLP, ISSR, etc. for the efficiency of cryopreservation. Until now, there are known studies concerning the genetic diversity of *Dianthus callizonus*, *D. giganteus* ssp. *banaticus*, *D. glacialis* ssp. *gelidus*, *D. henteri*, *D. nardiformis*, and *D. spiculifolius* using AFLP and RAPD technique [118,119].

These findings are also taken into account by other authors [24,41,120].

The developed in vitro propagation protocols can be applied by other countries to similar species, for either mass production or conservation purposes.

Anthropogenic pressure and changing environmental conditions will require in the future the use of in vitro cultures even more for propagation, conservation, biotransformation, obtaining cell lines (cell cultures) for the creation of new products, especially secondary metabolites that are currently used to replace synthetic substances in medicines, food additives (flavors, dyes, preservatives), insecticides, perfumes, etc. At the same time, we have to take into account the possibility of using in vitro culture for cultivation of native species in urban green spaces.

The development and improvement of in vitro techniques for the propagation and preservation of cells, tissues, and plant organs belonging to the studied species is necessary because they are a source of genes and for the amelioration of cultivated species.

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