In Vitro Cellular & Developmental Biology - Plant (2019) 55:635–646
https://doi.org/10.1007/s11627-019-10014-6

INVITED REVIEW

In vitro propagation of medicinal and aromatic plants: the case of selected Greek species with conservation priority

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Received: 24 September 2018 / Accepted: 26 August 2019 / Published online: 9 September 2019 / Editor: Praveen Saxena

Abstract

Worldwide, many medicinal and aromatic plants (MAPs) are still collected from the wild and only a small fraction of them are exclusively sourced from cultivation. This practice when performed non-sustainably threatens species and populations. Micropropagation of MAPs is a powerful tool to conserve rare, threatened, and valuable MAPs, and to massively produce high-value plant material for cultivation without seasonal constraints. In this study, the *in vitro* propagation protocols of 22 Greek native MAPs assigned with conservation priority were assessed (herbaceous perennials, bulbous, subshrubs, and trees), including 17 range-restricted plants and 5 taxa of Orchidaceae. For the latter, current micropropagation efforts include seed germination, callus induction, and protocorm formation for successful plantlet development; however, these propagation protocols are still fragmentary. For the rest ($n = 17$), a five-stage detailed procedure is outlined (plant material, establishment, proliferation, rooting, and acclimatization), while materials, treatments, and data per stage are shown comparatively and discussed. Emphasis is given on the selection and preparation of plant material obtained from nature for research, sustainable use, and *ex situ* conservation actions, and on their effectiveness for conservation purposes or mass production needs. The protocol effectiveness was calculated using a specific equation to estimate the potential number of acclimatized plants raised from a single explant within a year. All protocols can facilitate conservation, and almost half of them could be used for commercialization with high cost (five cases), intermediate cost (eight), or low cost (four), which enables their possible sustainable use.

Keywords Greek flora · *Ex situ* conservation · Sustainable exploitation · Propagation protocol effectiveness · Plant growth regulators

Introduction

Medicinal and aromatic plants (MAPs) have properties that are highly valued in the pharmaceutical, cosmetic, fragrance, food, and flavor industries, and many are widely cultivated, *e.g.*, *Lavandula* spp. (lavender), *Origanum vulgare* L. subsp. *hirtum* (Link) Iestw. (Greek oregano), *Rosmarinus officinalis* L. (rosemary), *Origanum majorana* L. (marjoram), *Matricaria chamomilla* L. (chamomile), *Salvia* spp. (sages), and *Melissa officinalis* L. (lemon balm). However, to date, the production of MAPs still relies to a large degree on wild collection (Lange 1998; Kupke *et al.* 2000), such as in Albania (Lange 1998), Hungary (Bernáth and Németh 2000), Slovakia (Kupke *et al.* 2000), and Turkey (Özhatay 1997). In Europe alone, the vast majority of > 1300 different MAPs used are still collected from the wild, while only 3 to 6% are exclusively sourced from cultivation. The practice of harvesting wild plants coupled with land conversion or habitat loss is currently threatening with extinction about 20 to 25% of MAPs (Lange 1996; Murch *et al.* 2004; Schippmann *et al.* 2006).

The uncontrolled harvesting in the Mediterranean, which dates back to antiquity, has been identified as a major threat for the populations of the MAPs, and it has led to several species being on the verge of extinction, such as the rosemary in Sardinia (Mulas and Mulas 2005), and *Gentiana acaulis* L. and *Arnica montana* L. in Croatia (Šatović 2004). At least 30% of the rare and threatened plants of Greece, many of which have medicinal and/or aromatic properties, actually

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suffer from uncontrolled wild collection (Krigas *et al.* 2014a). For example, the wild populations of the local Balkan endemic *Sideritis scardica* Griseb. (Greek mountain tea, 'Tsai Olympou') are already assessed as "Near Threatened" by the IUCN (International Union for the Conservation of Nature) Global Red List; *Sideritis sipylea* Boiss. (endemic to Greek East Aegean Islands and adjacent part of Anatolia, Turkey) and *Sideritis euboea* Heldr. (single-island endemic of the island of Evia, Greece) are both nationally assessed as "Endangered"; *Sideritis raeseri* Boiss. & Hledr. subsp. *attica* (Heldr.) Papan. & Kokkini (local endemic of Sterea Hellas) is assessed as "Vulnerable" (Phitos *et al.* 2009); and the populations of the Cretan endemic *Sideritis syriaca* L. subsp. *syriaca* (Cretan Mountain tea or Malotira) have severely declined during the last decades, which has led to a population monitoring program launched recently in Crete. Out of ten wild perennial taxa (species and subspecies) of the genus *Sideritis* traditionally traded in Greece for the preparation of mountain tea, only a couple of them are cultivated on a small scale (*S. scardica*, *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*), while cultivation of *S. syriaca* subsp. *syriaca* and *Sideritis clandestina* (Bory & Chaub.) Hayek subsp. *clandestina* has only recently started.

Furthermore, there is an increase in the electronic trade of range-restricted single-country endemic plants, among which the majority are MAPs and many of them are threatened with extinction. Examples include the Greek endemics *Origanum dictamnus* L. and *Origanum calcaratum* Juss. (Krigas *et al.* 2014b), and the Cypriot endemics *Origanum cordifolium* (Montbret & Aucher ex Benth.) Vogel, *Scutellaria cypria* Rech. f. subsp. *cypria*, and *Sideritis cypria* Post (Krigas *et al.* 2017).

Although domestication and cultivation of MAPs is the most sustainable method to exploit their valuable properties (Schippmann *et al.* 2006), knowledge regarding wild species of MAPs is still limited. For the latter, species-specific propagation and cultivation protocols are necessary for the industry and ornamental market to avoid draining wild populations. Therefore, sexual propagation is needed to raise new plants for conservation or breeding purposes, while asexual propagation is required for the production of genetically identical elite plants with high value. Multiplication using cuttings can be slow and labor intensive, rooting is often difficult (Gonçalves and Romano 2013), and domestication of rare and threatened plants could be problematic, especially when the availability of initial propagation material is usually restricted for these plants (Grigoriadou *et al.* 2011). *In vitro* propagation methods could help overcome these limitations and increase the probability of success (especially when threatened species are targeted), which would facilitate rapid multiplication of selected and valuable plant material for various uses without seasonal restrictions (Zuzarte *et al.* 2010).

In general, *in vitro* techniques for plant propagation (micropropagation) have major advantages over conventional methods: (*i*) For initial plant establishment, only a small amount of explant material is needed in a limited working space; (*ii*) propagation in a sterile environment ensures pathogen-free plants; (*iii*) large numbers of identical clones are raised, with genetic and physiological uniformity, and chemical consistency for commercial purposes, independent of seasonal variations (*e.g.*, from one explant, it is possible to produce over 1,000,000 plants in 1 yr); (*iv*) stock material can be securely maintained almost perpetually; and (*v*) the maintenance of mother plants is easier and safer given that one plant may be enough to take the explants needed for the establishment of the culture (George and Debergh 2008).

In this review, the current knowledge is summarized regarding the *in vitro* propagation of selected MAPs, herbaceous perennials, bulbous, subshrubs, and trees, which are native to Greece and are also assigned with conservation priority. The studies reviewed were sourced from (*a*) Scopus database, using the "*in vitro*" and "conservation" keywords in article titles and/or using the names of known scientists working with *in vitro* technology and Greek native plants; and (*b*) from other sources of grey literature such as faculty repositories, books, and conference proceedings. Among the *in vitro* studies retrieved, it was cross-checked whether each of the vascular taxa (species and subspecies) mentioned in these studies were assigned with (*i*) native status in Greece (Dimopoulos *et al.* 2013, 2016), (*ii*) conservation concerns due to restricted distribution range such as single-country Greek endemics and other range-restricted taxa (Dimopoulos *et al.* 2013, 2016); (*iii*) threatened or near threatened status, either nationally (Phitos *et al.* 1995, 2009; Tsiftsis and Tsiripidis 2016) or globally (see Global IUCN Red List website); (*iv*) MAP potential such as specific medicinal and aromatic properties, or specific ethnobotanical uses; and (*v*) national (Greek) protection status. After this strict selection procedure, only the cases of range-restricted species and subspecies (taxa) of Greece were included in the review, such as single-country endemics or endemics to Greek phytogeographical regions, or parts thereof, Balkan endemics or East-Mediterranean endemics, most of them threatened or near threatened and/or protected, which are currently considered as MAPs with phytochemical or ethnobotanical evidence. This included five additional Orchidaceae taxa (some with wider distribution range), with medicinal properties and ethnobotanical uses that are protected by the Greek legislation (Table 1, $n = 22$ taxa).

In vitro propagation of Greek native MAPs with conservation priority Micropropagation can provide a rapid method for the multiplication of conservation priority species. In this case, only a few plants may be available as stock plants, or the collection of plants and seeds from the wild may be minimized. Many institutes, organizations, and botanic gardens

Table 1 List of selected conservation priority medicinal and aromatic plants native to Greece reviewed in this study (alphabetically according to the family they belong to)

Family	Taxon	Range and endemism	Extinction risk-protection
Apocynaceae	^*Amsonia orientalis* Decne. in Jacquem.	Range-restricted East Mediterranean endemic (North-East Greece and parts in Turkey)	Critically endangered (1) protected
Asteraceae	^*Achillea occulta* Constantin. & Kalpoutz.	Range-restricted Greek endemic (South Peloponnese)	Vulnerable (2)
Asteraceae	**Staehelina petiolata* (L.) Hilliard & B. L. Burtt	Range-restricted Greek endemic (Crete)	
Boraginaceae	**Lithodora zahnii* (Halácsy) I.M. Johnst.	Range-restricted Greek endemic (South Peloponnese)	Vulnerable (3), protected
Campanulaceae	^*Campanula incurva* A. DC. in DC.	Range-restricted Greek endemic (Central mainland and some Aegean Islands)	Rare (3) protected
Dipsacaceae	**Lomelosia hymettia* (Boiss. & Spruner) Greuter & Burdet in Greuter & Raus	Range-restricted Greek endemic (Peloponnese, Sterea Hellas, West Aegean Islands)	
Fabaceae	**Anthyllis splendens* Willd.	Range-restricted Greek endemic (Crete and Cyclades)	Rare (3) protected
Fagaceae	!*Quercus trojana* Webb subsp. *euboica* (Pappaioannou) Chr. in Strid & Tan	Range-restricted Greek endemic (Evia)	Vulnerable (3), protected
Liliaceae	°*Muscari macrocarpum* Sweet	Range-restricted East Mediterranean endemic (islands of the Aegean and south-west Anatolia, Turkey)	Protected
Lamiaceae	^*Calamintha cretica* (L.) Lam.	Range-restricted Greek endemic (Crete)	Vulnerable (2), protected
Lamiaceae	**Origanum dictamnus* L.	Range-restricted Greek endemic (Crete)	Near threatened (1) or vulnerable (2), protected
Lamiaceae	^*Sideritis perfoliata* L. subsp. athoa (Papan. & Kokkini) Baden in Strid & Tan	Range-restricted Balkan endemic (also Anatolia in Turkey)	Protected
Lamiaceae	^*Sideritis raeseri* Boiss. & Heldr. in Boiss. subsp. *raeseri*	Range-restricted Balkan endemic	
Lamiaceae	^*Sideritis scardica* Griseb.	Range-restricted Balkan endemic	Near threatened (1)
Lamiaceae	^*Sideritis sipylea* Boiss.	Aegean endemic (East Aegean islands, Greece and Anatolia in Turkey)	Endangered (2)
Lamiaceae	^*Sideritis syriaca* L. subsp. *syriaca*	Greek endemic (Crete)	Declining populations
Orchidaceae	°*Anacamptis laxiflora* (Lam.) R.M. Bateman, Pridgeon & M.W. Chase subsp. *laxiflora*	Mediterranean	Protected
Orchidaceae	°*Ophrys apifera* Huds.	Mediterranean	Protected
Orchidaceae	°*Ophrys argolica* H. Fleischm.	Including two range-restricted Greek endemic subspecies and two east Mediterranean endemics	Vulnerable (1, 3) or near threatened (4), protected
Orchidaceae	°*Orchis mascula* (L.) L. subsp. *mascula*	European-South West Asiatic	Protected
Orchidaceae	°*Ophrys scolopax* Cav. subsp. *cornuta* (Steven) E. G. Camus	Mediterranean	Protected
Rosaceae	!*Malus florentina* (Zucc.) C.K. Schneid.	Balkan endemic (also Italy)	Vulnerable (3) protected

Plants' growth forms are marked with symbols, *i.e.*, ^ Herbaceous perennials; ° Bulbous; * Subshrubs; ! Trees. Distribution range and endemism follow Dimopoulos *et al.* (2013, 2016). Extinction risk categories are given according to (1) www.iucnredlist.org, (2) Phitos *et al.* (2009), (3) Phitos *et al.* (1995), or (4) Tsiftsis and Tsiripidis (2016). Protection status of taxa (species and subspecies) refers to the Greek national legislation

involved in the *ex situ* conservation of native plants apply micropropagation procedures, when needed, for maintenance and clonal reproduction of important species (Hartmann *et al.* 2002; Maloupa *et al.* 2008). Without efficient conventional propagation methods for such species, or due to limited available material, or decline of wild populations, or original anthropogenic habitat alterations, the micropropagation procedure may facilitate future needs to reinforce wild populations by raising adequately selected plants from different stocks (neopopulations). Currently, reintroduction programs in Greece are only performed on a pilot scale in Crete; these include conservation translocation actions following the guidelines of the International Union for the Conservation of Nature (IUCN) and exclusively rely on plants raised *in vivo* by seeds to ensure enriched species' genetic diversity (see http://www.care-mediflora.eu/).

In most MAPs, micropropagated plants are usually generated by the development of axillary buds or adventitious shoots (Gonçalves and Romano 2013), which was usually a five-stage procedure (Debergh and Maene 1981; George and Debergh 2008). In this section, *in vitro* propagation of 17 cases of Greek native MAPs with conservation priority, which includes herbaceous perennials, bulbous, subshrubs, and trees, was examined (Tables 2, 3, and 4).

Stage 0: Mother plant selection and preparation The selection of initial plant material is important for any culture establishment and to avoid infected stock mother plants. For the Greek

native MAPs with conservation priority, a specific protocol was reported regarding research on their sustainable use (Maloupa *et al.* 2008). In this process, healthy target plants were collected from the natural environment using a special collection permit issued by the Greek government for specialized botanical expeditions in various regions of the national territory, during which appropriate propagation material was collected (seeds, cuttings, rhizomes, tubers, and/or whole plants). Plant material was transferred to the Laboratory for the Conservation and Evaluation of Native and Floricultural Species (LCENFS), and the Balkan Botanical Garden of Kroussia (BBGK; which is part of the Institute of Plant Breeding and Genetic Recourses, Hellenic Agricultural Organization-DEMETER), where samples were sorted and each one assigned a unique "accession code." The specimens were taxonomically identified, and some were also documented with DNA barcoding. To allow *ex situ* maintenance of previously unknown MAPs, a geographic information systems (GIS) ecological profiling was used (Krigas *et al.* 2010, 2012). The ecological information extracted can illustrate the soil and climate profiles in the wild habitats of the targeted plants quantitatively and qualitatively. This information is used to keep mother plants in appropriate environmental conditions so that any differences in their behavior during the later stages will be primarily due to genotype expression. Both LCENFS and BBGK apply this protocol and specialize in *ex situ* conservation of the native plants of Greece. Mother plants of the collection of LCENFS and BBGK were widely used for the establishment of tissue culture for Greek native species with conservation priority (Grigoriadou *et al.* 2011; Samaras *et al.* 2012; Grigoriadou *et al.* 2014; Sarropoulou and Maloupa 2016a, b), while others were initiated by plant material collected directly from the wild (Kartsonas and Papafotiou 2007; Martini and Papafotiou 2013).

Stage I: Establishment of *in vitro* cultures Greek native MAPs with conservation priority are often rich in essential oils and phenolic compounds (Panagouleas *et al.* 2003; Hanlidou *et al.* 2012; Hanlidou and Lazari 2013), and glandular hairs or multicellular glands containing essential oils, which makes disinfection and effective establishment difficult (Grigoriadou *et al.* 2011; Martini and Papafotiou 2013; Sarropoulou and Maloupa 2017a). Explants were disinfected using 1.5 to 4% (*v*/*v*) sodium hypochlorite (NaOCl), shaken on a rotary shaker for 20 min and rinsed 3 times with sterile distilled water, depending on the period of the year (Kartsonas and Papafotiou 2007; Martini and Papafotiou 2013), and/or on the type of explant (Grigoriadou *et al.* 2011, 2014; Sarropoulou and Maloupa 2017a; Table 2). Pretreatment with ethanol and/or fungicides was usually effective in three difficult cases (Sarropoulou and Maloupa 2017a, b).

For culture initiation of the studied taxa, shoot tips or nodal segments from fresh annual growth was typically used (Table 2). Seeds were also used, after disinfection using 1.5 to 4% (*v*/*v*) NaOCl with the addition of three drops of Tween® 20 (Öz *et al.* 2008; Papafotiou *et al.* 2017). Special pretreatments were sometimes needed to overcome seed dormancy. This was the case of *Amsonia orientalis* Decne. in Jacquem., in which the use of 100 μM sodium nitroprusside (SNP) was necessary for successful *in vitro* germination (Öz *et al.* 2008).

Murashige and Skoog (MS) medium (Murashige and Skoog 1962) was used for shoot tips and nodal segments during the initiation phase of most Greek native MAPs with conservation priority, and was supplemented with 6-benzyladenine (BA), either alone or in combination with auxins such as indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and α-naphthaleneacetic acid (NAA) (Table 2). Woody Plant Medium (WPM; Lloyd and McCown 1980) was used for *Quercus trojana* Webb subsp. *euboica* (Papaioannou) Chr. in Strid & Tan, which is a woody plant with hyperhydricity problems (Kartsonas and Papafotiou 2007).

Establishment success of Greek MAPs with conservation priority *in vitro* varied from 13 to 90%. Even lower rates were considered sufficient for this stage, because the objective was not a high success rate, but to have pathogen-free cultures readily available. In cases of limited and valuable initial material, even one plantlet could be sufficient and after continuous subcultures, enough stock material (identical) could be produced (George and Debergh 2008); however, this is at the expense of the concomitant species genetic diversity, thus compromising conservation efforts.

Stage II: Proliferation *in vitro* For the proliferation of Greek native MAPs with conservation priority, MS proved to be the preferred basal medium (Table 3). In some cases, the used medium was supplemented with extra iron (Fe; Minas 2001; Grigoriadou *et al.* 2011). This need was observed primarily for species that naturally grow at high altitudes, which have been maintained at lower altitudes where cultivation temperatures are generally higher, even in *in vitro* studies. Under those elevated temperatures, sometimes Fe is not efficiently absorbed by microplants (Monteiro *et al.* 2000). The MS medium contains Fe in the chelate form of Fe-ethylenediaminetetraacetic acid (EDTA). However, given that the most effective form of chelated Fe is Fe-ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid) (EDDHA), substitution with Fe-EDDHA could provide better results (Molassiotis *et al.* 2003). For the woody species studied, hyperhydricity problems were observed when WPM was used (Kartsonas and Papafotiou 2007). Woody plant medium contains one-third to one-fourth the level of macroelements compared with MS, but it is a medium with a very good balance of macroelements and is often used for difficult cases of micropropagation, such as micropropagation of species of the Fagaceae family (Ostrolucká *et al.* 2007).

Table 2 Most optimal conditions for the *in vitro* establishment of selected native Greek medicinal and aromatic plants with conservation priority (alphabetically, excluding Orchidaceae plants)

Taxon	Type of explant	Disinfection	Medium	Plant growth regulators	Average establishment success (%)	Reference
Achillea occulta	Shoot tips	1.7% (*v/v*) NaOCl for 10 min	MS	4 μM BA + 0.5 μM IBA	73	Grigoriadou *et al.* (2011)
Amsonia orientalis	Seeds	SNP 100 μM overnight, 2% (*v/v*) NaOCl for 10 min	MS	Free	13	Öz *et al.* (2008)
Anthyllis splendens	Seeds	2–4% (*v/v*) NaOCl for 10 min	MS	4 μM BA	80	Papafotiou *et al.* (2017)
Calamintha cretica	Seeds	2–4% (*v/v*) NaOCl for 10 min	MS	Free	79	Papafotiou *et al.* (2017)
Campanula incurva	Shoot tips	1.5% (*v/v*) NaOCl for 13 min	MS	1 μM BA + 0.1 μM IBA	55	Grigoriadou *et al.* (2014)
Lithodora zahnii	Seeds	4% (*v/v*) NaOCl for 10 min	½ MS	Free	> 70	Papafotiou and Kalantzis (2009b)
Lomelosia hymettia	Seeds	2–4% (*v/v*) NaOCl for 10 min	MS	Free	80	Papafotiou *et al.* (2017)
Malus florentina	Nodal segments	2–4% (*v/v*) NaOCl for 10 min	MS	4 μM BA + 0.5 μM IBA	33–83	Martini and Papafotiou (2013), Martini *et al.* (2013)
Muscari macrocarpum	Bulblet	4–5% (*v/v*) NaOCl for 20 min	MS	9 μM BA + 2.5 μM NAA	> 80	Ozel *et al.* (2009)
Origanum dictamnus	Shoot tips	2% (*v/v*) NaOCl	MS	20 μM BA + 0.05 μM IBA	45	Minas (2001)
Quercus trojana subsp. *euboica*	Shoot tips/nodal stem pieces	3% (*v/v*) NaOCl	WPM	4.44 μM BA	70	Katsonas and Papafotiou (2007)
Sideritis perfoliata subsp. *athoa*	Seeds	1–2% (*v/v*) NaOCl for 15 min	½ MS	Free	60–70	Papafotiou and Kalantzis (2009a)
Sideritis raeseri subsp. *raeseri*	Shoot tips	2% (*v/v*) NaOCl for 15 min	MS	Free	> 60	Sarropoulou and Maloupa (2017b)
Sideritis scardica	Shoot tips	3% (*v/v*) NaOCl for 25 min	MS	1.1 μM BA + 0.05 μM IBA + 0.05 μM NAA + 0.1 μM GA_3	45–50	Sarropoulou and Maloupa (2016a)
Sideritis sipylea	Shoot tips	1.5% (*v/v*) NaOCl for 15 min	MS	Free	50	Samaras *et al.* (2012)
Sideritis syriaca subsp. *syriaca*	Shoot tips	1.5–2.5% (*v/v*) NaOCl for 13 min	MS	Free/2μM BA	50	Samaras *et al.* (2012)/Sarropoulou and Maloupa (2017a)
Staehelina petiolata	Shoots tips	1.5% (*v/v*) NaOCl for 15 min	MS	4–8 μM BA	90	Antonidaki-Giatromanolaki *et al.* (2006)

BA, 6-benzyladenine; *GA_3*, gibberellic acid; *IBA*, indole-3-butyric acid; *MS*, Murashige and Skoog medium (Murashige and Skoog 1962); *NAA*, α-naphthaleneacetic acid; *SNP*, sodium nitroprusside; *WPM*, Woody Plant Medium (Lloyd and McCown 1980)

In cases of equally effective results, the latter and their references are separated with stroke (/)

Table 3 Overview of the best media and plant growth regulators (PGRs) tested for proliferation (rate calculated in 4-wk periods) and shoot length reported for *in vitro* studies related to native Greek medicinal and aromatic plants with conservation priority (alphabetically, excluding Orchidaceae plants)

Taxon	Basic media	PGRs	Average proliferation rate	Shoot length (cm)
Achillea occulta	MS mod	5 μM BA	3.5	0.8
Amsonia orientalis	MS	4 μM BA	4.0	Not mentioned
Anthyllis splendens	MS	1 to 2 μM BA	3	Not mentioned
Calamintha cretica	MS	1 to 2 μM BA	3	Not mentioned
Campanula incurva	MS	8 μM BA	4.0	0.8
Lithodora zahnii	MS	1 μM BA + 1 μM Zea	1.85/3.35	0.8/0.4
Lomelosia hymettia	MS	Free	3	Not mentioned
Malus florentina	MS	4 μM BA + 0.4 μM IBA	3.1	1.2
Muscari macrocarpum	MS	9 μM Kin + 2.5 μM NAA	4.5	0.1–0.4 (bulblets)
Origanum dictamnus	MS mod	20 μM BA + 0.4 μM IBA	2.0	1.5
Quercus trojana subsp. *euboica*	WPM	4 μM BA	1.5	2.8
Sideritis perfoliata subsp. *athoa*	MS	2 μM BA	2.5	0.6
Sideritis raeseri subsp. *raeseri*	MS	2 μM Zea + 0.2 μM NAA + 11 μM α-tocopherol	3.0	2.9
Sideritis scardica	MS	1.5 μM BA	4.5	1.6
Sideritis sipylea	MS	1.5 μM BA + 0.15 μM IBA	3.3	1.7
Sideritis syriaca subsp. *syriaca*	MS	2 μM BA + 0.2 μM IAA	3.2	1.5
Staehelina petiolata	MS	8 μM BA	3.2	Not mentioned

BA, 6-benzyladenine; *GA_3*, gibberellic acid; *IBA*, indole-3-butyric acid; *kin*, kinetin; *NAA*, α-naphthaleneacetic acid; *IAA*, indole-3-acetic acid; *zea*, zeatin; *MS*, Murashige and Skoog medium (Murashige and Skoog 1962); *WPM*, Woody Plant Medium (Lloyd and McCown 1980)

For references related to specific taxa (species and subspecies), see Table 2

In the studies of Greek MAPs with conservation priority, different plant growth regulators (PGRs) were used. Use of cytokinins either alone or in combination with auxins (Table 3), with BA being the most common, was also reported for other MAPs (Gonçalves and Romano 2013). The use of BA was effective in most cases and resulted in high proliferation rates, as in *A. orientalis* (Öz *et al.* 2008), and *Campanula incurva* A. DC. in DC. (Grigoriadou *et al.* 2014) (Table 3). In other cases, such as *Achillea occulta* Constantin. & Kalpoutz. (Grigoriadou *et al.* 2011), *Malus florentina* (Zucc.) C.K. Schneid. (Martini and Papafotiou 2013), and *Sideritis perfoliata* L. subsp. *athoa* (Papan. & Kokkini) Baden in Strid & Tan (Papafotiou and Kalantzis 2009a), BA gave new shoots that were < 1.5 cm high, which were not capable of advancing to the next stage of rooting (Table 3). In those cases, stage II should have been followed by a special treatment in different media for shoot elongation (stage IIIa, Debergh and Maene 1981), which would result in inevitably increased production costs (George and Debergh 2008). Zeatin (zea) was used in the cases of *S. raeseri* subsp. *raeseri* and *Lithodora zahnii* (Halácsy) I. M. Johnst. when other PGRs were not effective enough. The combination of 2 μM of zea with 11 μM of α-tocopherol for *S. raeseri* subsp. *raeseri* reduced the severity of hyperhydricity problems that appeared when zea was used alone (Sarropoulou and Maloupa 2017b). Hyperhydricity was also observed in *L. zahnii* cultures when a high concentration of zea was used (Papafotiou and Kalantzis 2009b). This zea effect was counteracted by using high concentrations of sucrose and agar.

Somatic embryogenesis and regenerated plants from mature organs have been reported for endangered species, allowing genetic improvement and supporting conservation efforts (Litz *et al.* 2008; Martínez *et al.* 2019). However, this has not yet been reported in Greek native MAPs with conservation priority, probably due to limited research in this area in Greece.

Stage III: Rooting *in vitro* Before rooting *in vitro*, it may be necessary to allow time for shoots to grow to a suitable size (usually > 1 to 1.5 cm), depending on the species (Debergh and Maene 1981). In many Greek native MAPs with conservation priority, it seems that expanding the elongation treatment would have been probably useful leading to length increases > 1.5 cm (Table 3).

For the rooting of taxa reviewed in this research, MS was the usual medium, sometimes with macronutrients reduced by half, which was a typical procedure reported for other MAPs (Dias *et al.* 2002; Gonçalves and Romano 2013). For an effective micropropagation protocol, the addition of auxins (IBA in most cases) in concentrations of 2 to 10 μM proved to be effective, and resulted in a high percentage of rooting

Table 4 Overview of media and plant growth regulators (PGRs) used for rooting (in 4-wk subculture) and ready-plant acclimatization (calculated in 6 wk) as reported in *in vitro* studies related to native Greek medicinal and aromatic plants with conservation priority (alphabetically, excluding Orchidaceae plants)

Taxon	Rooting media (basic + PGRs)	Average rooting (%)	Average acclimatization success (%)	*Np*	Effectiveness (cp per mp)	Cost
Achillea occulta	MS (mod) + 20 μM IBA	12.5	33	3250	E/NE	HC
Amsonia orientalis	MS + 3 μM IAA	100	84	215,000	E/E	LC
Anthyllis splendens	½ MS + 5 μM IBA	90	> 90	16,000	E/E	IC
Calamintha cretica	½ MS + 5 μM IBA	90	> 90	16,000	E/E	IC
Campanula incurva	½ MS + 5 μM IBA	100	> 95	> 1,000,000	E/E	LC
Lithodora zahnii	MS + 1.5 μM IBA	93	> 85	42,500	E/E	IC
Lomelosia hymettia	½ MS + 5 μM IBA	90	> 90	16,000	E/E	IC
Malus florentina	½ MS + 30 μM IAA + 2 g l^{-1} activated charcoal	60	> 83	13,200	E/E	HC
Muscari macrocarpum	MS + 4 μM Kin + 5 μM NAA	100	100	750,000	E/E	LC
Origanum dictamnus	MS (mod), free	90	90	8230	E/NE	HC
Quercus trojana subsp. *euboica*	MS + 10 μM IBA	84	> 85	< 200	E/NE	HC
Sideritis perfoliata subsp. *athoa*	MS + 8 μM IBA + 4 μM NAA	53	> 85	1700	E/NE	HC
Sideritis raeseri subsp. *raeseri*	MS + 2.5 μM IBA	90	95	15,300	E/E	IC
Sideritis scardica	MS + 20 μM NAA	73	85	418,785	E/E	LC
Sideritis sipylea	MS + 8 μM IBA	80	> 95	38,200	E/E	IC
Sideritis syriaca subsp. *syriaca*	½ MS + 5 μM IBA + 2.5 μM NAA	90	97	30,100	E/E	IC
Staehelina petiolata	MS + 2 μM IBA	80	> 85	23,900	E/E	IC

IAA, indole-3-acetic acid; *IBA*, indole-3-butyric acid; *kin*, kinetin; *NAA*, α-naphthaleneacetic acid; *MS*, Murashige and Skoog medium (Murashige and Skoog 1962)

The protocols used are designated separately as effective (E) or not effective (NE) for conservation purposes (cp) or for mass propagation (mp), as well as high cost (HC), intermediate cost (IC), or low cost (LC). The potential number (*Np*) of acclimatized plants in 1 yr from one explant (the *Np* numbers given per taxon are rounded to the nearest hundred) is calculated as $N_p = PR^{n-1} \times R\% \times AC\%$, where PR is average best proliferation rate among treatments according to cited references (Table 3), including reported losses due to hyperhydricity; *n* is number of proliferation subcultures achieved in 1 yr (calculated in 4-wk periods); *n*−1 is given, to allow the average time needed for successful establishment; *R* is average rooting percentage (%, not in decimal values) according to cited references (calculated in 4-wk periods; Table 4); AC is average acclimatization success (%, not in decimal values) according to cited references (calculated in 6-wk periods; Table 4)

(Table 4). *Achillea occulta*, *M. florentina*, and *S. perfoliata* subsp. *athoa* showed poor rooting results. The addition of activated charcoal enhanced rooting of *M. florentina* (Martini and Papafotiou 2013). Even though these MAPs had a sufficient proliferation rate, the shoot length was short, which was probably the reason for their limited root induction. As previously highlighted, an elongation stage could prove to be beneficial (increasing the length to > 1.5 cm) for these taxa.

Stage IV: Acclimatization The acclimatization of Greek native MAPs with conservation priority mainly depended on the previous *in vitro* rooting stage (Table 4). For *C. incurva*, the micropropagation procedure proved to be the ultimate multiplication method, as it is estimated that > 1,000,000 plants can be produced, starting from one explant in a 1-yr period (Grigoriadou *et al.* 2014).

In the cases of conservation priority Greek native MAPs, in which root induction was > 50%, acclimatization success of the rooted plantlets was > 80% in a period of 4 wk, which was considered to be sufficient for an effective massive micropropagation protocol (Table 4). Only for *A. occulta* 33% survival was reported, which was due to limited *in vitro* rooting (Grigoriadou *et al.* 2011).

Assessment of protocol effectiveness An *in vitro* propagation protocol that can be broadly applied should generate thousands of acclimatized plants in a short period of successive subcultures (*e.g.*, Firoozabady and Gutterson 2003; Kaur and Sandhu 2015). In order to be able to compare the effectiveness of the different protocols produced in an integrated way, the number of successfully acclimatized plants for the Greek native MAPs with conservation priority that can be generated in 1 yr from one explant (*Np*) was calculated in the present study (Table 4), based on information reported in original sources (or calculated from data reported in them). To do this, the basic parameters that were taken into account were (*i*) best average

proliferation rate (*PR*) per studied species, as reported from different number of replicates, including reported losses due to hyperhydricity; (*ii*) proliferation subcultures achieved (*n*) per studied species in 1 yr (calculated in 4-wk periods), allowing the routinely needed time for successful establishment (*n-1*); (iii) average rooting percentage (*R*) per studied species (as reported from different number of replicates, calculated in 4-wk periods); and (*iv*) average acclimatization success rate per studied species (*AC*), according to cited references (calculated in 6-wk periods). All the abovementioned parameters (*i*, *ii*, *iii*, *iv*) represent specific rates with no units, and all refer to different numbers of plant individuals that potentially can be proliferated, rooted, and acclimatized, respectively, from a single explant per given species, if the best treatments reported in the reviewed studies are followed. Therefore, if these parameters are combined, the potential number of successfully acclimatized plants (N_p) of a given Greek native MAP species with conservation priority obtained from a single explant within 1 yr can be calculated using the following equation:

$$N_p = \mathrm{PR}^{n-1} \times R\% \times \mathrm{AC}\%$$

where

PR average best proliferation rate among treatments according to cited references (Table 3), including reported losses due to hyperhydricity;

n number of proliferation subcultures achieved in 1 yr (calculated in 4-wk periods); $n-1$ is given, which allows the average time needed for successful establishment;

R average rooting percentage (%, not in decimal values) according to cited references (calculated in 4-wk periods; Table 4);

AC average acclimatization success (%, not in decimal values) according to cited references (calculated in 6-wk periods; Table 4).

The calculation of N_p was introduced to allow quantitative comparison of the propagation protocols reviewed in terms of conservation purposes and mass production. The N_p assumes that there are no space limitations, meaning that all explants produced during subsequent subcultures will proceed to rooting with no time intervals, and those rooted plants will be immediately transferred for acclimatization. With these limitations in mind, in this study, a protocol was arbitrarily considered as "effective for conservation purposes" when > 100 plants yr^{-1} could be reproduced and as "effective for massive production" when 10,000 plants yr^{-1} could be reproduced. These threshold values reflect in an empirical way the amount of plants needed when conservation is the objective (*e.g.*, 100 individuals yr^{-1} raised from a limited and difficult to access initial material for selective population re-enforcement), or when routine massive production is considered for a given species (*e.g.*, 10,000 plants yr^{-1} from an elite material with desired characteristics for small scale commercial cultivation). With this perspective, effective protocols for conservation purposes were designated for all 17 Greek native MAPs with conservation priority reviewed in this study. For 13 MAPs, the protocols reviewed were also considered as effective for mass reproduction. However, for four of them (*A. occulta*, *O. dictamnus*, *Q. trojana* subsp. *euboica*, *S. raeseri* subsp. *raeseri*), the described methods were characterized as "not effective" in terms of mass reproduction, due to the long time required to generate a high number of acclimatized plants (Table 4).

Micropropagation is argued to be an expensive method of plant propagation, as it requires the use of specialized personnel and expensive infrastructure. When it is used for commercial mass propagation, the protocols followed should produce several hundreds of thousands of plants within a year (De Paoli *et al.* 1994). For example, cost-effective protocols are considered to be those resulting in 1,850,000 acclimatized plants for the industrial crop *Saccharum officinarum* L. (sugarcane; Kaur and Sandhu 2015), and 850,000 for *Ananas comosus* (L.) Merr., the pineapple crop (Firoozabady and Gutterson 2003). Therefore, if the above-described N_p is > 100,000, then the protocols could be applied commercially at low cost, especially for conservation important MAPs. If the N_p is < 100,000, these would result in high-cost reproduction schemes, while intermediate costs (balancing costs and benefits) could be attributed to $N_p > 15{,}000$ (Table 4). Following this perspective, it seems that five taxa could be multiplied at high cost (*A. occulta*, *M. florentina*, *O. dictamnus*, *Q. trojana* subsp. *euboica*, *S. perfoliata* subsp. *athoa*), eight taxa at intermediate cost (*Anthyllis splendens* Willd., *Calamintha cretica* (L.) Lam., *L. zahnii*, *Lomelosia hymettia* (Boiss. & Spruner) Greuter & Burdet in Greuter & Raus, *S. raeseri* subsp. *raeseri*, *S. scardica*, *S. sipylea*, *S. syriaca* subsp. *syriaca*, *Staehelina petiolata*), and only four of the taxa (*A. orientalis*, *C. incurva*, *Muscari macrocarpum* Sweet, *S. scardica*) could be massively multiplied at low cost (Table 4). However, given the conservation priority assigned for all the taxa of Table 4, their maintenance and proliferation *in vitro* are considered an important steppingstone towards conservation.

***In vitro* propagation of Orchidaceae MAPs of Greece with conservation priority** Agroalimentary and medicinal uses of orchids, for making salep or salepi (a flour made from the tubers of orchids) in particular, date back to ancient traditions in Greece and several species are involved (Kreziou *et al.* 2015), such as *Anacamptis* spp. (including *Anacamptis laxiflora* (Lam.) R.M. Bateman, Pridgeon & M.W. Chase subsp. *laxiflora*, Korakis and Vidakis (2016)), *Dactylorhiza* spp. and *Orchis* spp., especially *Orchis mascula* (L.) L. subsp. *mascula* (Kreziou *et al.* 2015). Additionally, flower parts of

Table 5 Overview of materials, techniques, optimal media, plant growth regulators (PGRs), and substances used during the establishment phase and protocorm formation, as reported in *in vitro* studies on native Greek medicinal and aromatic Orchidaceae with conservation priority (alphabetically)

Taxon	Type of explant	Disinfection	Medium	Plant growth regulators	Culture duration (months)	Germination/callus formation (%)	Protocorm formation (%)	Reference
Anacamptis laxiflora subsp. *laxiflora*	Mature seeds	1% (*v*/*v*) NaOCl for 5–10 min	SM-organic	25 μM kin + pineapple juice + 1 cube of potato	6	60–75	Not mentioned	Katsalirou *et al.* (2017)
Ophrys apifera	Mature seeds	10% (*v*/*v*) CaOCl for 15 min	Malmgren (1996)	Coconut milk	3	90	25	Kitsaki *et al.* (2004)
Ophrys argolica	Immature seeds	10% (*v*/*v*) CaOCl for 15 min	Malmgren (1996)	Pineapple juice	3	93	0	Kitsaki *et al.* (2004)
Orchis mascula subsp. *mascula*	Mature seeds	2% (*v*/*v*) H_2SO_4 for 5 min + 1% (*v*/*v*) NaOCl for 20 min	Orchimax	8 μM BA + 0.5 g L^{-1} active charcoal	3	5	5	Valletta *et al.* (2008)
Ophrys scolopax subsp. *cornuta*	Immature seeds	10% (*v*/*v*) CaOCl for 15 min	Malmgren (1996)	Coconut milk	3	58	20	Kitsaki *et al.* (2004)

BA, 6-benzyladenine; *kin*, kinetin; SM-organic (Katsalirou *et al.* 2017)

Orchis apifera Huds., *Orchis argolica* H. Fleischm., and *Orchis scolopax* Cav. subsp. *cornuta* (Steven) E. G. Camus may be rich in flavonoids and could be used as antioxidants (Karioti *et al.* 2008).

In Greece, the Orchidaceae family is currently represented by 141 taxa (species and subspecies), according to Dimopoulos *et al.* (2013, 2016), or by 193 taxa according to Tsiftsis and Tsiripidis (2016), and taxa in this family are nationally protected (Presidential Decree 67/1981, law 1335/1983, and law 2005/1992, which enacts CITES, known as the Convention on International Trade of Threatened Species).

There is a very limited amount of published data on the *in vitro* propagation of European terrestrial orchids (Valletta *et al.* 2008). Compared with tropical orchids, temperate ones are harder to propagate and cultivate (Ponert *et al.* 2011), and the few reports that exist lack detail on the laboratory techniques used (Katsalirou *et al.* 2017). In this context, propagation of Mediterranean orchids is poorly documented. There are just a few reports from Greece, although Greece is the most orchid-rich country in Europe based on the number of taxa per unit area (Georghiou and Delipetrou 2010).

Seed germination and callus formation Seed treatments using various disinfecting agents prior to establishment represent a vital step during micropropagation of orchids and serve a dual purpose: to disinfect the seeds and to break embryo dormancy (chemical scarification). The literature is contradictory as to the recommended duration of disinfection/scarification that leads to seed germination in various species (Katsalirou *et al.* 2017). A scarification time of only a few minutes in 1% (*v*/*v*) NaOCl seems optimal for germination of seeds with rather permeable coats, such as those of *A. laxiflora* subsp. *laxiflora* (Table 5). Due to dormancy induced with maturation of Orchidaceae seeds, non-mature seeds are preferable for *in vitro* culture (Malmgren 1996; Steele 1996). Kitsaki *et al.* (2004) reported different reactions of immature and mature seeds of bulbous Orchidaceae MAPs of Greece with conservation priority (Table 5). For MAPs of Greece of the genus *Ophrys* with conservation priority, mature seeds and a coconut-enriched medium were reported as a proper treatment for *in vitro* germination, although there were cases in which using immature seeds resulted in better germination results and callogenesis (Table 5).

Different culture media have been used for seed germination of bulbous Orchidaceae MAPs of Greece with conservation priority, and the addition of organic substances such as coconut milk, pineapple juice, or a cube of potato seem to be critical (Kitsaki *et al.* 2004; Valletta *et al.* 2008; Katsalirou *et al.* 2017). However, the reproducibility of these trials could be questioned due to the variable composition of these supplements. The duration of the germination/callus formation

stage from seeds was long, from 3 to 6 mo, and occasionally, the success was low (Table 5).

Protocorm formation and plantlet development In the native Greek Orchidaceae MAPs with conservation priority studied, protocorm formation compared with callogenesis was reported to be considerably lower, and this was true for both mature and immature seed cultures, especially on pineapple-enriched media (Kitsaki *et al.* 2004). Once the protocorms developed from callus tissues, they could easily continue to plantlets. Thus, the most crucial stage remains the differentiation of callus to form shoot and root meristems. This, for the native Greek Orchidaceae MAPs with conservation priority, was influenced by the genotype, varying from 0 to 55% in a 3-mo period, and was not proportional to the frequency of callogenesis (Table 5).

In the native Greek Orchidaceae MAPs with conservation priority studied, protocorms developed into plantlets with leaves and roots. Next, they were transferred to the same growing medium for 10 mo, which allowed minituber formation and were then successfully transferred for culture in pots (Kitsaki *et al.* 2004).

Conclusions

This review focused on 22 native MAPs of Greece with conservation priority (mostly range-restricted Greek endemics), for which there was available information regarding their *in vitro* propagation. The study reflects the current state of knowledge on this topic.

Regarding the effectiveness of *in vitro* propagation of Greek native species of Orchidaceae family with MAP potential and conservation priority, germination protocol improvements must be further investigated to decrease the time required to reach plantlet development. Although these data are rather fragmentary, they can facilitate and serve the future development of species-specific *in vitro* propagation protocols including shoot formation, rooting, and acclimatization. By that time, although the *ex situ* conservation can be investigated for these taxa, their sustainable exploitation and possible commercialization will still be compromised.

Despite the limited number of cases reviewed in this study, the protocols produced to date show that *in vitro* culture techniques are efficient not only for increasing the number of plants in cases in which other methods are insufficient or inadequate, but also for the *ex situ* conservation of Greek native species with conservation priority.

The protocols reviewed were assessed in terms of effectiveness either for conservation purposes or mass production needs with an equation produced in this study. While all of them were assessed as effective for conservation purposes, it seems that in more than half of the cases ($n = 13$), these are also effective for mass production, allowing their possible sustainable exploitation and commercialization. However, this can be done with high cost in five cases, with intermediate cost in nine cases, and with low cost only in three cases (*A. orientalis*, *C. incurva*, *M. macrocarpum)*.

Undoubtedly, a large majority of the Greek native MAPs with conservation priority has not been studied yet. However, at least for those taxa reported here, selected plant material in excellent hygienic condition can become readily available either for conservation purposes, reintroduction into the wild or reinforcement of wild populations, or for breeding trials and cultivation programs.

Acknowledgments The authors would like to thank the anonymous reviewers for their constructive comments. The work of Nikos Krigas, Katerina Grigoriadou, and George Tsoktouridis was supported by the project "Multifaceted Valorisation of single-country endemic plants of Crete, Greece, Tunisia and Rif, Morocco for sustainable exploitation in the agro-alimentary, horticultural-ornamental and medicinal-cosmetic sectors" (MULTI-VAL-END) which is funded through the ARIMNet2 2017 Joint Call by the following funding agencies: HAO-Demeter (Greece), MHESRT (Tunisia), and MESRSFC (Morocco); ARIMNet2 (ERA-NET) has received funding from the European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement no. 618127.

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