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Research Article

In vitro direct organogenesis of the Cretan dittany (*Origanum dictamnus* L.), an important threatened Greek endemic species

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Abstract

Dittany of Crete (*Origanum dictamnus* L.) is a threatened medicinal-aromatic plant of Lamiaceae family which is a local endemic to the Island of Crete, Greece. Its high culinary use and increasing demand in the pharma, perfumery, cosmetic and food industry along with its overexploitation from its natural habitat has threatened this species and has necessitated its large-scale production for industrial exploitations using advanced technologies. Micropropagation is considered a good tool for *ex situ* conservation of endangered species with reduced populations in the wild, low germination rates and low seed production. In this study, moderate germination percentages (40-41.38%) were exhibited for seeds after 40 days of culture at 21-23 °C in MS medium regardless of photoperiod regime (16h light/ 8h dark, 24h darkness), without significance difference. In the proliferation and rooting stage, three basal culture media (MS, WPM, Gamborg B5) were tested in combination with two concentrations of 6-benzyladenine (BA) (1.1, 2.2 μM) and indole-3-butyric acid (IBA) (0.125, 0.25 μM), all supplemented with 0.3 μM gibberellic acid (GA_3), 20 g L^{-1} sucrose and 6 g L^{-1} Plant Agar (pH: 5.8). The results showed that the MS medium + 2.2 μM BA + 0.25 μM IBA was the most effective treatment for micropropagation of shoot nodal explants in a single stage within a 30-day culture period, exhibiting 85% shoot formation, 1.8 new shoots/ explant 2.8 cm long with a 3.2 proliferation rate, 100% rooting, 16.5 roots/ rooted explant 2.1 cm long. Rooted plants obtained *in vitro* from MS medium enriched with 2.2 μM BA + 0.25 μM IBA gave 100% *ex vitro* survival rate on a peat: perlite (1:1 v/v) substrate mixture after 2 months in the greenhouse mist. In this study, an efficient *in vitro* propagation system of *O. dictamnus* is described for the first time through optimization of direct organogenesis stages (seed germination, proliferation, rooting, *ex vitro* acclimatization), as a means to facilitate domestication procedure, *ex-situ* conservation and future sustainable exploitation strategies, thus promoting wider usability of this local endemic with significant commercial potential.

Keywords: basal tissue culture media; conservation priority species; *ex situ* conservation; medicinal-aromatic plants; micropropagation; plant growth regulators; seed germination

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Introduction

Dittany of Crete (*Origanum dictamnus* L.) (Lamiaceae) is a unique aromatic perennial local endemic plant to the Island of Crete, Greece (Krigas *et al.*, 2015), assessed as Near Threatened (Bilz *et al.*, 2011) or Endangered (Kougioumoutzis *et al.*, 2021) against the IUCN criteria, and locally cultivated (Olivas *et al.*, 2020). It is protected by the Greek Presidential Decree 67/1981 and its traditional use as herbal medicine is supported by a European Medicines agency monograph (Krigas *et al.*, 2015; Olivas *et al.*, 2020). A preliminary assessment of the conservation status of endemic, endangered, medicinal and aromatic plants is imperative, at national, regional and international levels (IUCN 2001).

Dittany is an important species of high commercial potential due to its usage as a herbal tea (infusion, decoction) for tinctures, oral or cutaneous use (Liolios *et al.*, 2010; Chinou, 2013, Chishti *et al.*, 2013, EMA/HMPC, 2013) all around Greece, including the local markets of Crete island, traditional or modern shops and open-air market stalls (Hanlidou *et al.*, 2004; Liolios *et al.*, 2010). Regarding its culinary use in the food industry, dried leaves and extracts have been integrated into liqueurs, bitters, and fish sauces, while leaves and inflorescences to salads, sauces, and vermouth (Chishti *et al.*, 2013). Dittany of Crete is assessed as a safe and effective traditional herbal medicinal product due to its orexigenic, antioxidant, digestive and antimicrobial activities (EMA/HMPC, 2013). The essential oils of the plant, accumulated in its non-woody above ground parts, are rich in volatile compounds (1.5-2.5%) such as carvacrol, γ-terpinene, thymol and p-cymene (Liolios *et al.*, 2010). A phytochemical investigation in the polar extracts of the aerial parts of *O. dictamnus* revealed 15 secondary metabolites mainly depsides and other phenolic compounds constituents (Chatzopoulou *et al.*, 2010). Even though this plant is well-recognized in several countries, there are no available products in these countries that are sold on a commercial scale in the market, except in Greece (EMA/HMPC, 2013).

The majority of *Origanum* species exhibit low germination rates due to high seed dormancy including the seeds of oregano, which have a prolonged dormancy period and germinate poorly (Thanos *et al.*, 1995), therefore they have restricted dispersal (Farashah *et al.*, 2011). Based on the studies of Montezuma-De-Carvalho *et al.* (1984), the long-term storage (ca. -18°C) is an effective conservation method for *Origanum* species, as it ensures the safe conservation of seeds for at least a period of 8 years. Germination is also dependent upon the age of the seeds; old seeds germinate to a higher percentage than fresh ones, possibly as a result of the volatilization of the essential oils present on the nutlet coat (Theophrastus 1926).

Therefore, the application of biotechnological methods is needed for the propagation and conservation of *Origanum* species (Oluk and Çakir, 2009) including micropropagation (El-Beyrouthy *et al.*, 2015) in an attempt to promote and popularize this state-of-the-art advanced technology transfer among farmers, entrepreneurs and pharmaceutical industry (Leelavathi and Kuppan, 2013). *In vitro* propagation through tissue culture plays an important role in mass multiplication, plant improvement, plant breeding, regeneration of elite or superior clones, exchange of planting material, germplasm conservation, genetic manipulation, DNA barcoding, secondary metabolites production, drug extraction, delivery, targeting, as well as standardization, all of which are of paramount significance (Kumar and Bhardwaj, 2020). There is little information in the literature about the *in vitro* culture of *Origanum* species (Kumari and Saradhi, 1992; Socorro *et al.*, 1998; Minas, 2001; Goleniowski *et al.*, 2003; El-Beyrouthy *et al.*, 2015).

There is no previous report on the application of tissue culture for direct organogenesis of *O. dictamnus*. In this study several factors and parameters were taken into consideration including the effect of the photoperiod on seed germination, the basal culture medium in combination with plant growth regulators (PGRs) concentration on shoot proliferation and rooting and their impact on the final stage of *ex vitro* acclimatization. In the present study, an effective protocol was established, developed and optimized for the first time for the *in vitro* mass multiplication scale of *O. dictamnus* L., also known as dittany of Crete.

Materials and Methods

Botanical collections

The botanical collection was performed on the island of Crete (Greece) in early March of 2003 (1/3/2003) using a special permit of the Balkan Botanic Garden of Kroussia (BBGK), which is issued yearly by the Greek Ministry of Environment and Energy endorsing the provisions of the Convention of Biological Diversity, Nagoya Protocol and EU Directive 511/2014. Plant material composed of young individuals and cuttings was carefully collected from wild-growing populations of *O. dictamnus* L., found as rock-dwellers on a calcareous geological substrate in the so-called geographic site "Ebaros" at an altitude of 430 m above sea level, 51 km from the state of Iraklio of the Crete Island (northern Greece), avoiding damages for the wild plants. The collected plant material was then transferred to the facilities of BBGK - Institute of Plant Breeding and Genetic Resources in Thermi, Thessaloniki and received an International Plant Exchange Network accession number (GR-1-BBGK-03,2108) after taxonomical identification. The young plants collected from the wild habitats were transplanted in pots, placed outdoors on benches under shade without direct sunlight, wherein acclimatized *ex situ* for long-term conservation purposes. In early September of 2011, seeds collected from mother plants which were maintained *ex situ*. To desiccate, the seeds (n= 60 seeds, 0.008 g) were placed for 80 days in a dark chamber at 15 °C and relative humidity (RH) of 15%. The experimentation *in vitro* started at the end of November 2018 using 7-year-old seeds after long-term storage in sealed containers within a seed bank at 4 °C and RH < 5%.

In vitro seed germination

The germination capacity *in vitro* of 7-year-old seeds of *O. dictamnus* was investigated. The basal medium used was the MS (Murashige and Skoog, 1962) enriched with 20 g L^{-1} sucrose and 6 g L^{-1} plant agar (seed pre-treatment: hydration at dH_2O for 24h). Seeds were disinfected by immersion for 30 min in a Signum [BASF, Germany, active ingredients: 267 g Kg^{-1} boscalid and 67 g Kg^{-1} pyraclostrobin, formulation: water dispersible granule (WG)] fungicide solution (0.07 g/ 100 mL ddH_2O) followed by 70% ethanol (30 sec) and 5% NaOCl solution (15 min). Two photoperiod regimes were tested including 16h light/ 8h dark and 24h darkness, wherein 30 seeds were used in each regime. Seeds were incubated in a growth chamber at a temperature of 22 ± 1 °C. The defined germination criterion was the emergence of the radicle, while observations were taken between the 5th and 40th day. After germination, plants were sub-cultured every 4 weeks on the same medium until sufficient material was concentrated for further experimentation.

In vitro shoot proliferation-rooting and ex vitro acclimatization of rooted plantlets

The experimental material used for proliferation experiments was shoot single-node explants 1-1.5 cm long with two buds obtained from previous *in vitro* cultures. The effect of three basal culture media i.e. MS, Woody Plant Medium (WPM) (Lloyd and McCown, 1980) and Gamborg B5 (Gamborg *et al.*, 1968) in combination with two concentrations of 6-benzyladenine (BA) (1.1 and 2.2 μM) and two concentrations of indole-3-butyric acid (IBA) (0.125 and 0.25 μM) was studied. All six media were supplemented with gibberellic acid (GA_3), 20 g L^{-1} sucrose (Duchefa Biochemie) and 6 g L^{-1} Plant Agar (Duchefa Biochemie) (pH: 5.8) and autoclaved at 121 °C for 20 min. Explants were transferred into Magenta vessels (Baby food jars, 62.4 mm × 95.8 mm, 200 mL) containing 35 ml of medium. All cultures were maintained in a growth chamber under 16-h photoperiod (40 μmol m^{-2} s^{-1}) supplied by cool white fluorescent lamps and T 22 ± 2 °C. After 30 days of culture, shoot formation percentage (%), number of new shoots per explant, shoot length (cm), proliferation rate, hyperhydricity percentage (%), necrosis percentage (%), rooting percentage (%), root number per rooted explant and root length (cm) were recorded.

In late winter (early January), rooted plantlets derived from the six *in vitro* treatments were planted in multiple-hole propagation trays of 100 mL volume, in a substrate mixture consisting of peat (Terrahum, Klasmann): perlite at a 1:1 v/v ratio and transferred to a heated mist system in the greenhouse, at 16 ± 2 °C base temperature, 5-15 °C air temperature, 80-100% RH for 15 days and 50% reduced light intensity (thermal curtains). After this period RH was gradually reduced and light intensity was increased. On early spring (March), the survival rate was recorded and acclimatized plantlets were transplanted into 0.33 Lt (8x8x7 cm) larger volume pots containing a more enriched peat (TS2, Klasmann): perlite (Perflor) substrate mixture at a 3:1 v/v ratio, and placed on a greenhouse bench (T 17-24 °C and RH 55%-70%). After another 3 months (early summer, June), the plants were transplanted in 2.5 L volume pots in peat (TS2): perlite: soil (2:1:½ v/v) substrate mixture and transferred to the natural environment (outside greenhouse) under sprinkling irrigation and shading net conditions.

Statistical analysis

The experimental layout was completely randomized. The means were subjected to analysis of variance (ANOVA) and compared using the Duncan multiple-range test (P < 0.05) ± standard error (S.E.) using the statistical program SPSS 17.0 (SPSS Inc., Illinois, New York, USA). The experiment regarding the seed germination assay included two photoperiod regimes (16h light/ 8h darkness, 24h dark) and six culture incubation periods (5, 6, 9, 12, 32, 40 days) where measurements were recorded, thus the main effect of factors (photoperiod regime, days of culture) and their interaction was determined by General Linear Model/ 2-way ANOVA. The experiment regarding proliferation and rooting was a 3x2 factorial one with three basal culture media (MS, WPM, B5), and two different BA + IBA concentrations (1.1 μM + 0.125 μM, 2.2 μM + 0.25 μM), thus including 6 treatments with 20 repetitions/ treatment (5 explants/ vessel x 4 vessels/ treatment). The main effect of factors (basal culture medium type, BA + IBA concentration) and their interaction was determined by General Linear Model/ 2-way ANOVA).

Results

In vitro seed germination

In *O. dictamnus* L., germination was initiated on day 5 for seeds incubated in the light and on day 6 for those in the dark. After 40 days of culture, 40% and 41.38% germination rates were recorded under 24h darkness and 16h light/8h dark, respectively without significant difference. The germination rate reached its optimum 37.93% on day 12 in the light and 36.67% on day 32 in the dark. The t_{50} for seeds incubated in the light and in the dark was 7 and 11 days, respectively. There was a significant increase in the germination rate from 6.9% to 37.93% between the 5th and 12th day in the light while non-significant was the further increase in the germination rate (37.93-41.38%) from the 12th to the 40th day. Accordingly, germination rates followed a continuously increasing trend (0-36.67%) within the first 32 days of culture in 24h darkness whereas the slight increase noticed in the germination rate (from 36.67% to 40%) between the 32nd and 40th day was not significant. Within the 40 days, the germination rates were ranged between 6.90-41.38% in the light and 0-40% in the dark. Even though germination rates during the first 12 days were higher in the light (6.90-37.93%) than in the dark (0-30%), no differences were observed between the two photoperiod regimes for the later period of 32-40 days (36.67-41.38%). Photoperiod regime, culture days and their interaction significantly affected germination ($p < 0.001$) (Table 1, Figures 1A-D).

In vitro shoot proliferation-rooting and ex vitro acclimatization of rooted plantlets

MS and B5 media enriched with 2.2 μM BA + 0.25 μM IBA gave higher shoot formation percentages, 85% and 90% respectively. All 3 media tested regardless of BA + IBA concentration except MS + 1.1 μM BA + 0.125 μM IBA (0.8 new shoots per explant) exhibited a significantly higher number of new shoots per explant (1.4-1.8) (Table 2, Figure 2A). Shoot length (2.8 cm) and proliferation rate (3.2) were higher when MS medium was fulfilled with 2.2 μM BA plus 0.25 μM IBA (Table 2, Figure 2B).

Table 1. *In vitro* germination of 7-year-old *O. dictamnus* L. seeds stored in a cold chamber (4-5 °C, RH < 5%), after 40 days of culture in MS medium supplemented with 20 g L^{-1} sucrose and 6 g L^{-1} Plant Agar under two photoperiod regimes; 16h light/ 8h dark and 24h darkness

Days of culture	*In vitro* germination (%)	
	16h light/8 h dark (t_{50}= 7 days)	24h darkness (t_{50}= 11 days)
5th	6.90 d	0.00 e
6th	13.79 c	6.67 d
9th	31.03 b	10.00 cd
12th	37.93 a	30.00 b
32th	37.93 a	36.67 a
40th	41.38 a	40.00 a
p-values (General Linear Model/ 2-way ANOVA)		
Photoperiod regime (A)	0.000***	
Days of culture (B)	0.000***	
(A)*(B)	0.000***	

Means with the same letter in the two columns [*in vitro* germination (%)] are not statistically significantly different from each other according to the Duncan's multiple range test at p ≤ 0.05. ***p ≤ 0.001

Figure 1. *In vitro* seed germination of *O. dictamnus*; (A) Seeds before disinfection and initial establishment; (B) Germinated seedling after 20 days of incubation in the MS medium PGRs-free under 24h darkness (etiolated, top-up figure) and 16h light/ 8h dark (green colored); (C, D) Growth of derived seedlings in the MS medium PGRs-free after 40 days of culture

Hyperhydricity was evident on WPM containing 1.1 μM BA + 0.125 μM IBA (10%) or 2.2 μM BA + 0.25 μM IBA (35%) whereas non-hyperhydrized explants were observed in the other two media. Phenomena of necrosis were observed only to the 10-20% of explants cultured in WPM and B5 media supplemented with the highest concentration of BA + IBA (Table 2). MS enriched with 2.2 μM BA + 0.25 μM IBA exhibited a higher rooting percentage (100%), root number per rooted explant (16.5) and root length (2.1 cm) being statistically significant compared with the other treatments (40-60% rooting percentages, 2.8-7.3 roots/ rooted explant, 0.9-1.5 cm root lengths) (Table 2, Figure 2B). Among the three basal culture media and regardless of BA + IBA concentrations, the MS was the most effective medium as exhibited better proliferation and rooting results without apparent hyperhydricity symptoms to the explants, WPM was the least effective due to hyperhydricity occurrence to the 10-35% of explants, while B5 medium showed moderate performance yields between the other two media (Table 2). Therefore, the MS medium enriched with 2.2 μM BA + 0.25 μM IBA was selected as the most preferred combination treatment for further experimentation (Table 2, Figure 2B). Survival rates of 87.5-100% were recorded for rooted plants derived *in vitro* from MS medium, 62.5-83.3% from B5, and 50-75% from WPM, irrespective of BA + IBA concentrations. *Ex vitro* survival rate 100% was recorded for rooted microshoots derived from MS + 2.2 μM BA + 0.25 μM IBA (Table 2, Figure 3).

Table 2. Effect of three basal culture media in combination with two BA + IBA concentrations, all enriched with 0.3 μM GA_3 on parameters characterizing *in vitro* shoot formation and rooting of *Origanum dictamnus* after 30 days of culture, and *ex vitro* survival rate after 30 days in the mist

Basal culture medium	BA (μM)	IBA (μM)	Shoot formation (%)	Number of new shoots/ explant	Shoot length (cm)	Proliferation rate	Hyperhydricity (%)	Necrosis (%)	Rooting (%)	Root number / rooted explant	Root length (cm)	*Ex vitro* survival (%)
MS	1.1	0.125	35.0 ± 9.6 c	0.8 ± 0.2 b	1.1 ± 0.1 c	0.6 ± 0.2 c	0.0 ± 0.0 c	0.0 ± 0.0 b	50.0 ± 17.3 b	3.4 ± 0.4 c	1.1 ± 0.1 cd	87.5 ± 12.5 ab
WPM	1.1	0.125	70.0 ± 12.9 ab	1.4 ± 0.2 a	1.2 ± 0.1 c	0.9 ± 0.2 c	10.0 ± 4.1 b	0.0 ± 0.0 b	60.0 ± 11.6 b	5.6 ± 0.5 bc	1.5 ± 0.1 b	75.0 ± 8.3 abc
B5	1.1	0.125	70.0 ± 5.8 b	1.4 ± 0.2 a	0.7 ± 0.0 d	1.1 ± 0.2 c	0.0 ± 0.0 c	0.0 ± 0.0 b	60.0 ± 8.2 b	7.3 ± 0.8 b	1.4 ± 0.1 bc	83.3 ± 9.6 ab
MS	2.2	0.25	85.0 ± 9.6 a	1.8 ± 0.2 a	2.8 ± 0.2 a	3.2 ± 0.3 a	0.0 ± 0.0 c	0.0 ± 0.0 b	100.0 ± 0.0 a	16.5 ± 2.2 a	2.1 ± 0.2 a	100.0 ± 0.0 a
WPM	2.2	0.25	70.0 ± 5.8 b	1.6 ± 0.1 a	1.5 ± 0.1 b	1.0 ± 0.0 c	35.0 ± 5.0 a	20.0 ± 8.2 a	40.0 ± 8.2 b	2.8 ± 0.1 c	1.1 ± 0.1 cd	50.0 ± 0.0 c
B5	2.2	0.25	90.0 ± 5.8 a	1.7 ± 0.2 a	1.1 ± 0.1 c	1.7 ± 0.2 b	0.0 ± 0.0 c	10.0 ± 5.8 ab	40.0 ± 18.3 b	3.4 ± 0.4 c	0.9 ± 0.1 d	62.5 ± 12.5 bc
P-values (General Linear Model/ 2-way ANOVA)												
Basal medium type (A)			*0.097 ns*	*0.361 ns*	*0.000****	*0.000****	*0.000****	*0.075 ns*	*0.089 ns*	*0.000****	*0.008***	*0.008***
BA + IBA conc. (B)			*0.004***	*0.003***	*0.000****	*0.000****	*0.001***	*0.008***	*0.743 ns*	*0.010***	*0.755 ns*	*0.143 ns*
(A)(B)*			*0.031**	*0.110 ns*	*0.000****	*0.000****	*0.000****	*0.075 ns*	*0.014**	*0.000****	*0.000****	*0.096 ns*

Means ± standard error (S.E.) with the same letter within columns are not statistically significantly different from each other according to the Duncan's multiple range test at $p \leq 0.05$. n.s.: $p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$

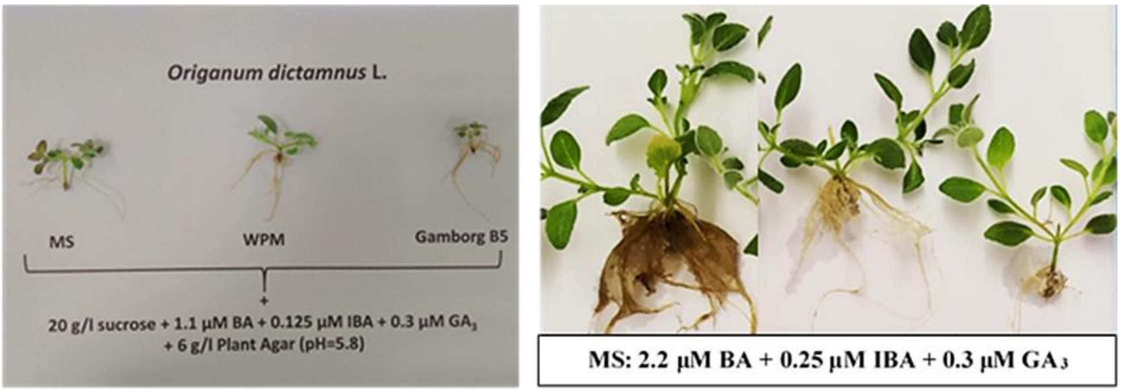

Figure 2. *In vitro* shoot proliferation and rooting of *O. dictamnus* explants after 30 days of culture; (A) Effect of three different basal culture media (MS, WPM, B5) supplemented with 1.1 μM BA, 0.125 μM IBA and 0.3 μM GA_3; (B) Maximum response under the combined effect of 2.2 μM BA, 0.25 μM IBA and 0.3 μM GA3 on MS basal nutrient medium

Figure 3. *Ex vitro* acclimatization and vegetative growth of rooted *in vitro O. dictamnu*s plantlets after transplantation to larger volume pots (100 mL, 0.33 L, 1 L, 2.5 L) from the mist, to non-heated greenhouse bench and finally to the external environment in the nursery

Discussion

In vitro seed germination

In the present study, *O. dictamnus* seeds cultured in MS medium exhibited higher germination rates; 37.93-41.38% at 16h light/ 8h dark or 36.67-40% at 24h darkness between the 32nd and the 40th day, without significant difference. In a previous study conducted on the same plant species *O. dictamnus*, similar germination rates (54.4-59.2%) are reported for seeds cultured either in darkness or under diurnally alternating light and dark conditions, in specific the daily length of the photoperiod being gradually decreased from 11.6 to 10.8 h during the experimentation period and the daily temperature fluctuation has been between 15 and 20 °C at the beginning, being gradually shifted to 11-16 °C by the end of the 30-day period (Thanos and Doussi, 1995). Regardless of the photoperiod regime and taking into consideration the minimum culture period required for maximum response, the germination ability of *O. dictamnus* seeds was significantly higher (37.93%) after 12 days of culture under 16h light/ 8h dark conditions. Different results to ours i.e., higher germination rate (100%) but longer culture period in MS medium (21 days) were reported for *O. minutiflorum* O. Schwarz & Davis seeds (Özkum, 2007). In the current study, the t50 for *O. dictamnus* seeds incubated at 16h light/ 8h dark and 24h darkness was 7 and 11 days, respectively. On the contrary, higher t50 values were reported for *O. dictamnus* seeds incubated either in the 16h light/ 8h dark (12.3 days) or in complete (24h) darkness (13.5days) by Thanos and Doussi (1995) than those obtained in our study (16h light/ 8h dark: 7 days, 24h darkness: 11 days), however in both studies, t50 values were lower in the 16h light/ 8h dark and higher in the complete (24h) darkeness. According to Fournaraki (2010), germination of *O. dictamnus* seeds is optimum at 10 and 15ºC with the highest t_{50} recorded in 5.7 days at 10 °C (germination onset 3-6 days and completion in 12-17 days). A 5-month storage period of *O. dictamnus* seeds at a low temperature of -20 °C had little effect on the time course of germination, reducing slightly the germination rate, therefore seeds are characterized by orthodox storage behavior as they maintained their viability (Fournaraki, 2010). The same perhaps applies to this study with *O. dictamnus* as well, because seeds before disinfection and initial establishment *in vitro* were stored for at least 7 years in the cold chamber of the seed bank under 4 ºC and RH < 5% conditions. In accordance with our findings, in *O. syriacum,* a close related Cretan endemics, the seeds without any pretreatment either in fluctuating temperature (15/20 °C) or at room temperature resulted in very close germination percentages (23.7% and 23.5%, respectively), whereas increased germination percentages (74-84%) were obtained after pretreatment with GA_3 (Ahmed, 2018), which reached to its maximum competence (> 90%) by pre-chilling seeds in light conditions and fluctuating temperature for eight weeks (Figuérédo *et al*., 2005). Therefore, it seems that the moderate germination rates of *O. dictamnus* achieved in this study can be attributed to dormancy due to unfavorable conditions (i.e. higher temperature than required, absence of fluctuating temperature and diurnally photoperiod conditions, longer previous cold storage at 4 ºC), which could be released by a variety of promontory treatments, including pre-chilling, light, GA_3 which are effective in Lamiaceae family species, thus promoting germination (Takano *et al*., 1990).

In vitro shoot proliferation-rooting and ex vitro acclimatization of rooted plantlets

Tissue culture efforts on *Origanum* spp. for *in-vitro* plant regeneration mostly used MS medium supplemented with different PGRs or other additives. Other media formulations like B5 medium, Nitsch and Nitsch medium (Nitsch and Nitsch, 1969), Phillips and Collins (L2) medium (Phillips and Collins, 1979) were used to test the explants' response for regeneration. Various PGRs have been used in tissue culture of *Origanum* species including cytokinins such as BA, 2-ip, zeatin, adenine sulphate and TDZ for shoot organogenesis applied either alone or in combination with auxins (IBA, IAA, NAA or 2,4-D) (Tejvathi and Padma, 2012; Sevindik *et al*., 2017). In the case of *O. dictamnus*, among the three basal media and the two BA + IBA concentration combinations tested, the MS medium fortified with 2.2 μM BA and 0.25 μM IBA promoted better proliferation and rooting of explants after 30 days of culture. Our results regarding the superiority of the MS medium for shoot proliferation are consistent with those recorded in several other *Origanum* species including *O. onites* (Atar and Çölgeçen, 2019), *O. syriacum* L. (El-Beyrouthy *et al*., 2015; Abdallah *et al*., 2017), *O. ehrenbergii* (El-Beyrouthy *et al*., 2015), *O. majorana* L. (Korkor *et al*., 2017), *O. sipyleum* (Sevindik *et al*., 2017), *O. vulgare* (Sajina *et al*., 1997; Leelavathi and Kuppan, 2013), *O. minutiflorum* (Özkum, 2007) and *O. vulgare* x *applii.* (Goleniowski *et al*., 2003). Regarding BA as the preferred cytokinin type better stimulating proliferation of *O. dictamnus*, a previous conducted study supports the suitability of 4.4

μM BA for multiple shoot production in a different *Origanum* species (*O. bastetanum*) (Socorro *et al.*, 1998). In this study, the addition of 2.2 μM BA + 0.25 μM IBA + 0.3 μM GA_3 in the MS medium promoted best the micropropagation of *O. dictamnus*. Our results related to optimum concentrations of PGRs for maximal shoot proliferation are in harmony with those conducted in other *Origanum* species such as *O. vulgare* (8.8 μM BA) (Leelavathi and Kuppan, 2013), *O. vulgare* x *applii* (low BA + NAA concentrations) (Goleniowski *et al.*, 2003), *O. majorana* (4.4 μM BA) (Korkor *et al.*, 2017), and *O. vulgare* (4.4 μM BA + 2.5 μM IBA) (Sajina *et al.*, 1997). The positive effect of BA on multiple shoot induction and growth could be attributed to the fact that cytokinins (mainly BA) inhibit apical dominance stimulating axillary shoots formation and foliar development of shoot tip cultures (Buah *et al.*, 2010).

In accordance with our findings where 85% proliferation and 100% rooting occurred within a 30-day period in a single micropropagation stage under the MS + 2.2 μM BA + 0.25 μM IBA treatment, Goleniowski *et al.* (2003) also reported rooting (100%) in shoot multiplication medium supplemented with BA (0.28 μM) + NAA (0.53 μM) for *O. vulgare* after 60 days of culture. On the other hand, there are several reports in other *Origanum* species showing that rooting was enhanced only by adding an auxin in the basal medium including 2.5 μM IBA in *O. sipyleum* L. (Oluk and Çakır, 2009), 7.38 μM IBA in *O. sipyleum* (Sevindik *et al.*, 2017), 1.1 μM NAA in *O. acutidens* (Hand.-Mazz.) Ietswaart (Yildirim, 2013), and 4.56 μM IAA in *O. syriacum* (Arafeh *et al.*, 2003). Indeed, the chemical composition of the basal medium seems to play a key role in the rooting ability of *O. dictamnus* microshoots based on previous studies in other *Origanum* species including *O. syriacum* L. in full-strength MS + 4.92 μM IBA or half-strength MS + 5.71 μM IAA (Abdallah *et al.*, 2017) or full-strength MS medium PGR's-free (El-Beyrouthy *et al.*, 2015), *O. vulgare* subsp. *hirtum* (half MS + 2.5 μM IBA) (Zayova *et al.*, 2019), *O. vulgare* (half-stength B5 + 1 μM IBA or NAA) (Kumari and Saradhi, 1992) and O. *ehrenbergii* (full-strength MS, PGR's-free) (El-Beyrouthy *et al.*, 2015).

Rooted *O. dictamnus* plants obtained *in vitro* from the MS medium enriched with 2.2 μM BA + 0.25 μM IBA gave an optimum *ex vitro* survival rate of 100%. Among the three basal media, irrespective of BA + IBA concentration combinations, the MS was the most effective medium (87.5-100%), the WPM the least effective (50-75%) and the B5 of intermediate effectiveness (62.5-83.3%). Similarly, several studies are pointing out the easiness of acclimatization and gradual hardening of rooted microshoots to the *ex vitro* environment in some other *Origanum* species including *O. sipyleum* L. (48-98%) (Oluk and Çakır, 2009), *O. majorana* L. (70%) (Korkor *et al.*, 2017), and *O. syriacum* L. (93%) (Abdallah *et al.*, 2017) based on the high survival rates achieved ranging from 48% to 93%, depended on the constituents and their ratio in the substrate mixture. In the studied *O. dictamnus*, the peat: perlite substrate mixture (1:1) used was effective for the acclimatization and adaptation stage of the plantlets after two months in the greenhouse mist.

Conclusions

In the present study, an efficient *in vitro* propagation system of *O. dictamnus,* a local endemic and endangered species to the Island of Crete (Greece) is described for the first time. This study highlights the development and achievements made for the micropropagation of Cretan dittany using shoot nodal explants. It is clearly illustrated that between the three basal culture media (MS, WPM, B5), and the two BA + IBA combination concentrations (1.1+0.125, 2.2+0.25 μM) tested, there is a strong inter-correlation and interaction. Among the combination treatments tested, the MS basal medium + 2.2 μM BA + 0.25 μM IBA was the most cost- and time-effective one for the micropropagation of *O. dictamnus* in a single-one procedure stage combining proliferation and rooting within a 30-day culture period. The results indicate the important potential of *in vitro* propagation to be used for large scale multiplication of *O. dictamnus* allowing thereby its cultivation and contributing to the sustainable use of its biological diversity.

Authors' Contributions

Conceptualization: VS and KG; Data curation: VS; Formal analysis: VS; Funding acquisition: KG; Investigation: VS and KG; Methodology: VS and KG; Project administration; KG; Resources: EM and KG; Software: VS and KG; Supervision: KG; Writing - original draft: VS; Writing - review and editing: VS, EM and KG. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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