Seed germination, micropropagation from adult and juvenile origin explants and address of hyperhydricity of the Cretan endemic herb *Calamintha cretica*

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Abstract

The optimum range of temperature for germination (96-100%) of *Calamintha cretica*, an herb with potential pharmaceutical and horticultural uses, was 15 to 20 °C, with 10 and 30 °C cardinal temperatures. Storage up to one year did not affect germination. The effect of zeatin (ZEA), 6-benzyladenine (BA), kinetin, and 6-γ-γ-(dimethylallylamino)-purine added in MS medium at concentrations from 0.0 to 8.0 mg L^{-1} was tested for shoot proliferation of both adult- and seedling-origin nodal explants at first- and sub-culture. Both explant types responded similarly during *in vitro* culture. At cytokinin concentrations up to 1 mg L^{-1} explant response was high (over 85%) but shoot number per explant was low (1.2-2.2). Increasing cytokinin from 2.0 to 8.0 mg L^{-1} resulted to an analogous decrease of explant response and shoot length, and an increase of shoot number, particularly when ZEA or BA was used (5.0-6.6 shoots per explant, 0.5-1.0 cm long) with simultaneous though increase of hyperhydricity (up to 50%). The addition of 0.1 mg L^{-1} naphthaleneacetic acid into the 8.0 mg L^{-1} BA medium almost eliminated hyperhydricity and increased explant response, while the increase of agar concentration from 8.0 to 12.0 g L^{-1} eliminated hyperhidricity and induced the highest shoot proliferation (93-95% explant response, 11.2-12.3 shoots per explant, 0.8-1.0 cm long). Microshoots and microshoot clusters rooted (88-96%) on half-strength MS medium either hormone free or supplemented with 1 to 4 mg L^{-1} indole-3-butyric acid. Plantlets survived at 80% to 100% after *ex vitro* acclimatization in peat: perlite 1:1 (v/v).

Keywords: agar; auxin and cytokinin concentration; *in vitro* rooting; Mediterranean plant; seed ecophysiology

Introduction

Calamintha cretica L., Lamiaceae, syn. *Clinopodium creticum* (L.) Kuntze, *Melissa cretica* L., *Satureja cretica* (L.) Briq., is a vulnerable endemic herb of Crete (Greece) that grows at limestone rocks, stony slopes and canyons (100-1.800 m a.s.l.) with low maquis (Phitos *et al.*, 2009). The plant has up to 30 cm long, horizontally spreading shoots, and small oval, pale green-gray, fragrant (between mint and oregano) leaves, covered by dense hairs and distinct glands. It blooms in May (mainly) until August, in corrugated inflorescence

with 3-4 small, pale lilac flowers. The leaves contain essential oil, its major compounds being piperitenone, piperitone oxide and p-menthane compounds, and thus it could be used in perfumery and in the pharmaceutical industry (Karousou *et al.*, 1996). Piperitenone oxide has been shown to have anticarcinogenic properties on human colon cancer cells (Nakamura *et al.*, 2014), while the essential oil of *C. nepeta* subsp. *nepeta* and subsp. *glandulosum* were found to have antifungal and insecticidal activity (Debbabi *et al.*, 2020). In Crete, mostly, it is used as herbal tea. The plant could also be introduced in Landscape Architecture, as a ground cover for urban and peri-urban gardens, parks and green roofs, similarly to the related species *C. nepeta* that has been suggested for such uses (Caneva *et al.*, 2013; Casalini *et al.*, 2017).

There are no reports found in the literature on propagation of the species, apart from a preliminary publication of ours, where it's micropropagation was studied starting from adult plants (Vlachou *et al.*, 2017b). This work revealed hyperhydricity problems that prevented proliferation from reaching a satisfactory level. Hyperhydricity is a morphological and physiological disorder of *in vitro* growing plants (Chakrabarty *et al.*, 2006), which results in loss of their ability to grow normally, practically reduces the proliferation potential, affects the quality of micropropagated plantlets, hyperhydrated shoots do not root, and plantlets have difficulties in *ex vitro* acclimatization (Ziv, 1991a; Hazarika, 2006). Several factors have been shown responsible for hyperhidricity, such as gelling agent, cytokinins (Ivanova and Van Staden, 2011), ammonium ions (Ivanova and Van Staden, 2008) and ventilation of the cultures (Ivanova and Van Staden, 2010).

The use of seedling-origin explants for in vitro cultures initiation usually results in high proliferation rate in Mediterranean species (Papafotiou *et al.*, 2013; Papafotiou and Martini, 2016). Further, propagation by seed enhances genetic diversity that is desirable when native plants are used for natural landscape restoration, and contributes to the selection of genotypes with high pharmaceutical value (Sarasan *et al.*, 2011).

Climatic changes characterized by temperature increasing at a rather rapid rate (Solomon *et al.*, 2007) are expected to have a significant impact on plant biodiversity and distribution (Pearson and Dawson, 2003; Lavergne *et al.*, 2006; Thuiller *et al.*, 2006). Germination of endemic species, which may be more restricted to particular temperature requirements, are expected to be more negatively affected than widely distributed species, and thus germination conditions of such species should be investigated.

Thus, with the incentive to introduce *C. cretica* to the horticultural and pharmaceutical industry, as well as to prevent it from extinction, in the present work, we studied a) the seed ecophysiology of the species, and b) it's micropropagation starting from either seedlings or adult plants. Specifically, we investigated a) the effect of seed-storage period and germination temperature on *C. cretica* germination ability and b) the effect of type and concentration of plant growth regulators and agar concentration on *in vitro* shoot proliferation and rooting, and elimination of hyperhydricity problems, in order to obtain an efficient micropropagation protocol of the species.

Materials and Methods

Seed germination

Seeds were collected in August 2014, from three *C. cretica* plants kindly provided to us (as rooted stem cuttings, 4-month old, in April 2013) by the Mediterranean Agronomic Institute of Chania, Crete. The plants were grown in a heated glasshouse of the Laboratory of Floriculture and Landscape Architecture, Agricultural University of Athens (37°58'58.0"N, 23°42'19.2"E). The seeds were left on the laboratory bench for 3 days to dry and then were stored in the dark at room temperature.

Fifteen days after harvest, and after 6 and 12 months of storage, seeds were tested for germination. After surface-sterilization, with 15% (v/v) commercial bleach (4.6% w/v sodium hypochlorite), with 1-2 drops of Tween 20 (polyxyethylenesorbitan monolaurate, MERCK), for 10 min, followed by four rinses (3 min each) with sterile distilled water, seeds were put in 9-cm Petri dishes with 20 ml of half-strength medium, at eight different temperatures (5, 10, 15, 20, 25, 30, 35, and 40 °C) and long days (16-h cool white fluorescent light

37.5 µmol m^{-2}·s^{-1}/8-h dark). Germination was defined after the emergence of at least 2 mm long radicle (International Seed Testing Association, 1999) and T$_{50}$ was defined as time for 50% seed germination.

Micropropagation

Seedling origin explants. Fifteen days after the completion of germination, seedlings were transferred to full-strength medium for further growth to be used as explant source for *in vitro* cultures. Seedling culture lasted six weeks and then shoot tip and single node explants were excised (2-3 explants from each seedling).

Adult origin explants.

Shoot tip and single node explants (0.6 cm long) derived from a *vitro* culture on hormone-free (Hf) medium. The stock *in vitro* cultures were initiated from shoot tip explants excised from the three adult plants (see above) grown in the greenhouse, and cultured on a medium supplemented with 1 mg L^{-1} 6-benzyladenine (BA) (Vlachou *et al.*, 2017b). The stock cultures were proliferated with a number of subcultures of shoot tips and single nodes on the initiation medium followed by one subculture on Hf MS (Murashige and Skoog, 1962) medium.

Effect of cytokinin type and concentration on shoot multiplication.

Shoot tip and single node explants of either seedling or adult origin were cultured on medium either hormone-free or supplemented with various cytokinins types, i.e., zeatin (ZEA), BA, kinetin (KIN), and 6-γ-γ-(dimethylallylamino)-purine (2IP) and concentrations. i.e., 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mgL^{-1}. One subculture followed, where shoot tip and single node explants were subcultured on the same fresh medium as that each one of which had originated.

Effect of plant growth regulators and agar concentration on hyperhydricity.

Aiming to address hyperhydricity that occurred in media with high cytokinin concentration, firstly was tested the addition of auxin into the cytokinin medium and secondly the increase of agar concentration. Therefore, single node explants were cultured on a medium either hormone-free or supplemented with 0.5, 1.0, 2.0, 4.0 or 8.0 mg L^{-1} BA and 0.1 or 0.5 mg L^{-1} naphthaleneacetic acid (NAA), in all possible combinations. In a following experiment, a medium supplemented with 8.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA that was solidified with 8.0 g L^{-1} agar was compared concerning shoot multiplication and hyperhydration with a medium supplemented with 8.0 mg L^{-1} BA and solidified with either 8.0 or 12.0 g L^{-1} agar.

In vitro rooting and ex vitro acclimatization.

Microshoots 2.0-2.5 cm long were put for rooting on half-strength medium either hormone-free or supplemented with 0.5, 1.0, 2.0 or 4.0 mg L^{-1} indole-3-butyric acid (IBA) for six weeks. In addition, clusters of microshoots were put for rooting on hormone-free half-strength medium.

Ex vitro acclimatization and establishment.

Rooted microshoots after being rinsed thoroughly with running tap water to remove growth medium were transferred to 500 ml containers (eight plantlets per container), on peat (High-more with adjusted pH up to 5.5-6.5, Klasmann-Delimann Gmbh, Geeste, Germany) and perlite (particles diameter 1-5 mm, Perloflor, ISOCON S.A., Athens, Greece) substrate 1:1 (v/v), covered for seven days with transparent plastic wrap (SANITAS, Sarantis S.A., Greece) and placed for one week in a growth chamber (20 °C and 16-h cool white fluorescent light 37.5 µmol.m^{-2}.s^{-1}/ 8-h dark photoperiod). Then the containers were uncovered and transferred in a heated glasshouse (37°58'58.0"N, 23°42'19.2"E), in the mist (spraying 15 s per 15 min from May to September and per 30 min from October to April; substrate temperature 22 °C maintained by thermostatically controlled electric heating cable), where they remained for 10 days and then they were placed on a greenhouse bench for 30 more days at the end of which data for acclimatization were recorded. After that the plants were

transplanted singly in 500 ml plastic pots with peat: perlite 2: 1, v/v and fertilized monthly with 2.0 g L^{-1} complete water-soluble fertilizer (Nutrileaf 60, 20-20-20; Miller Chemical and Fertilizer Corp., Hanover, PA, USA). Data on plant establishment ware recorded four months after.

Growth medium, in vitro culture conditions and data recording.

MS medium was used in all *in vitro* cultures. The medium contained 30 g L^{-1} sucrose except for seed germination that 20 g L^{-1} sucrose were used. All media were solidified with 8 g L^{-1} agar, except in hyperhydration experiments where 12 g L^{-1} agar were also used. The medium pH was adjusted to 5.7 before agar and autoclaving (121 ºC, 20 min). *In vitro* cultures were carried out in 100 ml Sigma glass vessels covered by Magenta B-Caps, with 25 ml medium (four explants per vessel), maintained at 25 ºC with a 16-h photoperiod at 37.5 µmol m^{-2} s^{-1}, provided by cool-white fluorescent lamps.

Data were recorded after 40 d of culture. Data for shoot tip and nodal explants were merged because both explant types responded similarly in all treatments applied. In shoot multiplication experiments, were recorded data on (a) explant response percentage to form normal shoots (NS), possibly coexisting with hyperhydrated shoots, or hyperhydrated shoots (HS) only, (b) NS and HS number per explant and (c) shoot length of NS, for explants that produced simultaneously NS and HS. In rooting experiments rooting percentage and root number and length were recorded.

The rate of plantlet survival at ex *vitro* acclimatization was recorded 40 d after transfer at the greenhouse, and data on plant establishment four months later.

Statistical analysis.

In all experiments, the completely randomized design was used and the significance of the results was tested by one- or two-way analysis of variance (ANOVA). Arcsin transformation of % data was not applied. The treatment means were compared by Student's *t* test at p≤0.05 (JMP 13.0 software, SAS Institute Inc., Cary, NC, 2013, USA). The number of replicates per treatment differed between experiments and is shown on the data tables.

Results and Discussion

Germination

Immediately after harvesting, *C. creticum* seeds germinated at 100% and in a short period of time (12-14 days) at 15 or 20 °C, without any pretreatment, in 16 h light, while germination was quite high at 10 and 25 °C (88% and 82%, respectively), as well (Table 1). Seeds stored for six months were germinated equally well compared to the shortly harvested ones, while seeds stored for 12 months had slightly reduced germination at 10 °C that was shown to be the low cardinal temperature for germination (Table 1). Germination was fastest at 20 °C (T_{50}=2), while at 10 °C T_{50} was quite increased (8-10), independently of storage period (Table 1). Cardinal temperatures for germination were defined at 10 °C and 30 °C and germination was prevented at 5 °C and 35 °C or higher temperatures (Table 1).

Germination percentages achieved were very high, similar to those we found for the close related species *Calamintha nepeta* (Vlachou *et al.*, 2019), although other researchers have reported lower germination performances for the latter (Benvenuti and Bacci, 2010; Casalini *et al.*, 2017). The high germination observed soon after seed harvest and for at least one year after, without any seed pretreatment, indicates absence of dormancy, something that characterizes relative species as well, i.e. *C. nepeta* (Vlachou *et al.*, 2019), *C. vulgare* (Angelova *et al.*, 1994) and *C. sandaliotica* (Mattana *et al.*, 2016), a number of other Lamiaceae plants of Crete *Origanum dictamnus, Sideritis syriaca* ssp. *syriaca, Salvia pomifera* ssp. *pomifera* (Thanos and Doussi, 1995), and a number of other Mediterranean species, as *Arbutus unedo, A. andrachne, A. × andrachnoides*

(Bertsouklis and Papafotiou, 2013), *Dianthus fruticosus* (Papafotiou and Stragas, 2009), *Sideritis athoa* (Papafotiou and Kalantzis, 2009a), *Lithodora zahnii* (Papafotiou and Kalantzis, 2009b).

Table 1. *In vitro* germination of *C. creticum* seeds at temperatures shown, after 1[z], 6, and 12 months of storage at room temperature

Storage (months)/ Germination temp. (°C)	Germination (%)	T_{50}[zz] (d)	Time (d) for full germination
1/5	0.0 ± 0.0 d[zzz]	-	0.0 ± 0.0 d
1/10	88.0 ± 3.7 b	8	16.0 ± 0.3 a
1/15	100.0 ± 0.0 a	4	14.0 ± 1.1 b
1/20	100.0 ± 0.0 a	2	12.0 ± 0.6 c
1/25	82.0 ± 5.8 b	4	14.0 ± 0.6 b
1/30	62.0 ± 8.0 c	2	12.0 ± 0.9 c
1/35	0.0 ± 0.0 d	-	0.0 ± 0.0 d
1/40	0.0 ± 0.0 d	-	0.0 ± 0.0 d
6/5	0.0 ± 0.0 d	-	0.0 ± 0.0 d
6/10	86.0 ± 4.0 b	10	16.0 ± 0.7 a
6/15	98.0 ± 2.0 a	4	16.0 ± 0.6 a
6/20	96.0 ± 4.0 a	2	16.0 ± 0.5 a
6/25	82.0 ± 3.7 b	4	14.0 ± 1.1b
6/30	68.0 ± 3.7 c	0	16.0 ± 1.1 a
6/35	0.0 ± 0.0 d	-	0.0 ± 0.0 d
6/40	0.0 ± 0.0 d	-	0.0 ± 0.0 d
12/5	0.0 ± 0.0 e	-	0.0 ± 0.0 d
12/10	80.0 ± 3.0 c	8	16.0 ± 0.6 a
12/15	98.0 ± 1.3 a	6	16.0 ± 0.5 a
12/20	98.0 ± 1.3 b	2	14.0 ± 1.0 b
12/25	76.0 ± 1.6 c	4	14.0 ± 1.1 b
12/30	62.0 ± 2.5 d	4	12.0 ± 0.6 c
12/35	0.0 ± 0.0 e	-	0.0 ± 0.0 d
12/40	0.0 ± 0.0e	-	0.0 ± 0.0 d
Significance of 2-way ANOVA			
$F_{Temperature}$	-	-	-
$F_{Storage}$	-	-	-
$F_{Temperature \times storage}$	*	***	***

[z] 1 month: actually, seeds were put for germination 15 days after harvest

[zz] T_{50} is defined as time for 50% seed germination

[zzz] Mean (±SE) separation in columns by Student's *t* test at $p \leq 0.05$ *, ***: significant at $p \leq 0.05$, $p \leq 0.001$, respectively.
n=5, 20 seeds/Petri dish (total 100 seeds per treatment)

The temperatures 15-20 °C were found optimum for germination for other Mediterranean species of the Lamiaceae family as well, i.e. *C. nepeta, S. syriaca* ssp. *syriaca, Coridothymus capitatus, Origanum vulgare* subsp. *hirtum, Satureja thymbra, Phlomis brevibracteata, P. cypria* ssp. *occidentalis* (Thanos and Doussi, 1995; Thanos *et al.*, 1995; Kadis and Georghiou, 2010; Vlachou *et al.*, 2019), and for the Mediterranean species of Fabaceae *Anthyllis barba-jovis* (Morbidoni *et al.*, 2008; Trigka and Papafotiou, 2017). For other Mediterranean species as *Dianthus fruticosus, Globularia alypum* and the three *Arbutus* species found in Greece 15 °C was the most favorable temperature for germination (Papafotiou and Stragas, 2009; Bertsouklis and Papafotiou, 2010, 2013), while for *Sideritis pungens, S. chamaedryfolia* and *S. athoa* the most appropriate germination temperature was slightly higher than 20 °C (Papafotiou and Kalantzis, 2009a; Estrelles *et al.*, 2010). Although it is usual for Mediterranean plant species to present germination optimum at 15 °C to 20 °C,

four Mediterranean geophytes of the genus *Muscari* (Liliaceae) germinated at even lower temperatures (optimum at 10 to 15 °C, Doussi and Thanos, 2002) and *L. zahnii* at 10 °C (Papafotiou and Kalantzis, 2009a).

Optimum temperature for germination was not affected by storage period, on the contrary to *C. nepeta* seeds that germinated better at 15 °C if stored for one year and at 20 °C if they were shortly harvested (Vlachou *et al.*, 2019). The cardinal temperatures for germination of *C. creticum* were found the same to *C. nepeta, S. pomifera subsp. pomifera* (Cretan sage), *Salvia fruticosa* native to Eastern Mediterranean regions and the Canary Islands, and *Salvia officinalis* native to the Mediterranean region but naturalized in many places throughout the world (Come, 1993; Thanos and Doussi, 1995; Vlachou *et al.*, 2019).

Temperature is one of the most important environmental factors controlling germination (Probert, 2000) that allows the seeds to avoid harsh environmental conditions for seedling establishment. It is usual for Mediterranean plant species to germinate at relatively low temperatures, because the low water availability in the Mediterranean ecosystems forces the seeds to germinate during autumn when the rainy season starts and the temperatures are cool.

The changes of climatic conditions, with temperatures increasing at a rather rapid rate (Solomon *et al.*, 2007), are expected to have a significant impact on biodiversity (Pearson and Dawson, 2003) and plant distribution (Lavergne *et al.*, 2006; Thuiller *et al.*, 2006). Germination of endemic species, which may be more restricted to particular temperature treatments, could be more negatively affected than widely distributed species, and thus studies on seed ecophysiology of such species are of special importance.

Shoot proliferation as affected by cytokinin type and concentration
Data for shoot type and single node explants were pulled because both explant types responded in a similar way in both first and sub-culture in the proliferation medium. Over 80% of the explants, independent of being seedling- or adult-origin, responded producing 1.2 to 1.4 shoots per explant at average, in both first- and sub-culture on Hf-medium (Table 2 and 3, Figure 1A). The addition of cytokinins in the medium at concentrations up to 1 mg L^{-1} (low concentrations) resulted in equally high explant response without though a considerable increase of shoot number. Increasing cytokinin concentration over 1 mg L^{-1} (2.0 to 8.0 mg L^{-1}, high concentrations) resulted to an analogous decrease of explant response and shoot length, simultaneously with an increase of shoot production and hyperhydrycity, particularly when 4.0 and 8.0 mg L^{-1} ZEA or BA were used (Table 2 and 3, Figure 1B, C, D, E). In these two highest concentrations of ZEA and BA the rate of explants that responded producing only hyperhydric shoots (Figure 1F) was quite high (over 15% to the first culture and over 20% in the sub-culture), while the rest of the responded explants apart from normal shoots formed 2 to 3.5 at average hyperhydric shoots as well (Table 2 and 3, Figure 1G). The number of shoots per explant was highest (4.6 to 6.6 at average) when 8 g L^{-1} ZEA or BA were used (Figure 1A, B, F, G), but a significant number of these shoots (1.8 to 3.5 at average) were hyperhydric (Figure 1F, G). Longest shoots in general were produced on the Hf-medium or the media with low cytokinin concentration, while the shortest shoots (shorter than 1 cm at average) were produced when 8 mg L^{-1} ZEA, BA or 2iP were used (Table 2 and 3).

C. cretica responded to MS medium and cytokinins in a very similar way with *C. nepeta* (Vlachou *et al.*, 2019), while the endemic of Mt Taygetos (South Peloponnese, Greece) *Nepeta camphorata* was found to proliferate quite successfully on Hf-MS medium (Darras *et al.*, 2020). A number of Mediterranean Lamiaceae and other xerophytes proliferated better at lower BA concentrations, 0.25 to 0.5 mg L^{-1}, compared to higher ones, 1.0 to 4.0 mg L^{-1} (Papafotiou *et al.*, 2017), while other Lamiaceae such as *Mentha piperita* and *Ocimum gratissimum* (Saha *et al.*, 2010, 2012) were found to have best response in terms of shoot formation at higher concentration of BA (1.0 to 2.0 mg L^{-1}).

Cytokinins has been shown to induce hyperhydricity in a number of plant species during *in vitro* proliferation depending on cytokinin type and concentration (Kataeva *et al.*, 1991; Ivanova and Van Staden, 2008; Ravanfar *et al.*, 2014; Vlachou *et al.*, 2019). Often hyperhydricity problems have been reported in the micropropagation of Mediterranean herbaceous or small-shrubby species (Bertsouklis *et al.*, 2003; Papafotiou and Kalantzis, 2009a; Trigka and Papafotiou, 2017; Vlachou *et al.*, 2017a, 2017b; Vlachou *et al.*, 2019). To

address hyperhydricity problems induced by cytokinin could be difficult, particularly in cases where cytokinin is needed at high concentrations for efficient proliferation. Reduction of medium-cytokinin concentration or the use of a different type of cytokinin led to the reduction of hyperhydricity (Sharma and Mohan, 2006; Papafotiou and Kalantzis, 2009a; Liu *et al.*, 2017) often though with simultaneous reduction of shoot production (Vlachou *et al.*, 2019).

Table 2. Effect of cytokinin type and concentration on shoot proliferation from seedling-origin explants excised from microshoots produced either on MS medium (I, first culture) or on the same medium as that tested for multiplication (II, subculture)

Cytokinin type	Cytokinin conc (mg·L⁻¹)	I (first culture)					II (subculture)				
		Shooting (%)	Shooting-hyperhydrated shoots only (%)ʸ	Mean NShᵀ number	Mean NSh length (cm)	Mean HShᵀᵀ numberˣ	Shooting (%)	Shooting-hyperhydrated shoots only (%)ʸ	Mean NSh number	Mean NSh length (cm)	Mean HSh numberˣ
Hfᵀᵀᵀ	-	92.9 ± 2.2 aˣ	0.0 ±0.0 j	1.4 ± 0.1 g	2.3 ± 0.2 d	0.0 ± 0.0 jk	92.9 ± 1.3 ab	0.0 ±0.0 j	1.4 ± 0.1 hi	3.3 ± 0.4 abc	0.0 ± 0.0 i
ZEA	0.25	88.9 ± 1.7 ab	0.0 ±0.0 j	1.9 ± 0.3 def	2.5 ± 0.2 cd	0.0 ± 0.0 jk	91.1 ± 2.1 abc	0.0 ±0.0 j	1.6 ± 0.2 ghi	4.0 ± 0.9 a	0.0 ± 0.0 i
	0.5	88.3 ± 1.2 ab	0.0 ±0.0 j	1.7 ± 0.2 efg	1.9 ± 0.3 de	0.0 ± 0.0 jk	88.9 ± 0.9 abc	0.0 ±0.0 j	1.5 ± 0.1 ghi	3.4 ± 0.2 abc	0.2 ± 0.1 h
	1.0	88.9 ± 1.5 ab	0.0 ±0.0 j	2.0 ± 0.3 def	2.6 ± 0.5 cd	0.0 ± 0.0 jk	90.0 ± 2.5 abc	0.0 ±0.0 j	1.6 ± 0.2 ghi	2.7 ± 0.5 cde	0.5 ± 0.1 g
	2.0	74.9 ± 1.8 de	8.2 ± 0.4 h	1.7 ± 0.2 efg	1.3 ± 0.2 efg	0.5 ± 0.1 f	71.8 ± 1.7 def	8.4 ± 0.4 i	3.0 ± 0.4 abc	1.6 ± 0.2 ghi	1.2 ± 0.1 e
	4.0	56.2 ± 1.6 hi	15.3 ± 0.7 c	1.8 ± 0.1 efg	0.8 ± 0.1 hij	0.9 ± 0.1 cd	54.0 ± 2.7 hij	21.4 ± 1.2 d	2.9 ± 0.4 bcd	0.9 ± 0.1 ijk	1.8 ± 0.1 c
	8.0	47.1 ± 1.6 jk	21.3 ± 0.6 a	3.1 ± 0.3 ab	0.6 ± 0.1 ij	2.0 ± 0.1 a	47.2 ± 1.9 jk	28.4 ± 1.0 b	3.3 ± 0.4 ab	0.6 ± 0.1 k	2.7 ± 0.1 b
BA	0.25	83.3 ± 4.0 bc	0.0 ±0.0 j	2.1 ± 0.4 cde	3.2 ±0.8 bc	0.0 ± 0.0 jk	83.1 ± 1.5 bcd	0.0 ±0.0 j	2.0 ± 0.3 efg	3.6 ± 0.5 ab	0.0 ± 0.0 i
	0.5	77.8 ± 4.5cde	0.0 ±0.0 j	2.2 ± 0.3 cd	1.8 ± 0.2 def	0.1 ij	88.9 ± 2.7 abc	0.0 ±0.0 j	2.3 ± 0.2 def	1.8 ± 0.2 fgh	0.2 ± 0.1 h
	1.0	66.7 ± 2.0 f	0.0 ±0.0 j	1.8 ± 0.2 efg	2.0 ± 0.2 de	0.3 ± 0.1 gh	77.8 ± 1.1 cde	0.0 ±0.0 j	1.9 ± 0.2 fgh	2.3 ± 0.4 efg	0.6 ± 0.1 g
	2.0	60.3 ± 1.9 gh	13.3 ± 0.8 de	1.6 ±0.2 efg	1.4 ± 0.1 efg	0.8 ± 0.1 d	60.4 g± 2.0 hi	10.0 ± 0.3 h	2.3 ± 0.2 def	1.0 ± 0.1 ijk	1.4 ± 0.1 d
	4.0	52.2 ± 1.9 ij	16.8 ± 0.8 b	2.6 ± 0.2 bc	1.0 ± 0.1 ghi	1.2 ± 0.1 b	50.0 ± 1.5 ijk	22.8 ± 0.8 c	2.9 ± 0.4 bcd	0.8 ± 0.1 jk	1.9 ± 0.1 c
	8.0	43.8 ± 2.0 k	20.6 ± 0.6 a	3.0 ± 0.2 ab	0.5 ± 0.1 j	2.0 ± 0.1 a	40.0 ± 1.5 k	30.3 ± 1.2 a	3.5 ± 0.3 a	0.6 ± 0.1 k	3.1 ± 0.1 a
KIN	0.25	94.4 ±1.7 a	0.0 ±0.0 j	1.8 ± 0.3 efg	4.1 ± 0.3 a	0.0 ± 0.0 jk	100.0 ± 0.0 a	0.0 ±0.0 j	1.2 ± 0.2 i	3.7 ± 0.2 ab	0.0 ± 0.0 i
	0.5	83.0 ± 2.7 bc	0.0 ±0.0 j	1.5 ± 0.3 fg	3.9 ± 0.4 ab	0.0 ± 0.0 jk	83.3 ± 3.5 bcd	0.0 ±0.0 j	1.4 ± 0.1 hi	3.2 ± 0.3 bcd	0.0 ± 0.0 i
	1.0	77.8 ± 2.2 cde	0.0 ±0.0 j	1.4 ± 0.1 g	3.2 ± 0.3 bc	0.0 ± 0.0 jk	80.8 ± 2.5 bcd	0.0 ±0.0 j	1.2 ± 0.1 i	3.1 ± 0.2 bcd	0.0 ± 0.0 i
	2.0	79.2 ± 2.8 cd	0.0 ±0.0 j	1.5 ± 0.2 fg	1.5 ± 0.2 efg	0.2 ± 0.1 hi	71.5 ± 3.1 def	8.3 ± 0.3 i	1.7 ± 0.1 fgh	1.5 ± 0.2 hij	0.5 ± 0.1 g
	4.0	64.7 ± 2.3 fg	10.2 ± 0.7 g	1.4 ± 0.2 g	1.3 ± 0.1 efg	0.5 ± 0.1 f	67.4 ± 2.4 efg	11.1 ± 0.6 h	2.0 ± 0.2 efg	1.5 ± 0.2 hij	0.8 ± 0.1 f
	8.0	60.0 ± 1.5 gh	12.3 ± 0.6 ef	2.0 ± 0.2 def	1.1 ± 0.2 ghi	0.7 ± 0.1 e	61.1 ± 2.1 ghi	14.6 ± 0.5 f	2.1 ± 0.2 efg	1.5 ± 0.2 hij	1.2 ± 0.1 e
2IP	0.25	86.9 ±1.2 ab	0.0 ±0.0 j	1.5 ± 0.2 fg	4.2 ± 0.4 a	0.0 ±jk	83.3 ± 3.0 bcd	0.0 ±0.0 j	1.4 ± 0.2 hijk	3.1 ± 0.4 bcd	0.0 ± 0.0 i
	0.5	83.3 ± 2.1 bc	0.0 ±0.0 j	1.6 ± 0.2 efg	3.8 ± 0.4 ab	0.0 ± 0.0 jk	88.9 ± 2.6 abc	0.0 ±0.0 j	1.5 ± 0.2 hijk	2.2 ± 0.2 efg	0.0 ± 0.0 i
	1.0	83.3 ± 1.8 bc	0.0 ±0.0 j	1.6 ± 0.3 efg	3.5 ± 0.5 ab	0.0 ± 0.0 jk	88.9 ± 2.7 abc	0.0 ±0.0 j	1.7 ± 0.3 fgh	2.4 ± 0.3 def	0.0 ± 0.0 i
	2.0	73.1 ± 1.9 ef	4.2 ± 0.3 i	1.5 ± 0.2 fg	1.2 ± 0.1 efg	0.5 ± 0.1 e	75.1 ± 1.5 def	8.4 ± 0.4 i	2.0 ± 0.2 efg	1.9 ± 0.3 fgh	0.6 ± 0.1 g
	4.0	64.4 ± 3.0 g	11.4 ± 0.6 fg	1.6 ± 0.2 efg	0.9 ± 0.1 ghi	0.9 ± 0.1 cd	64.9 ± 1.8 fgh	12.8 ± 0.6 g	2.2 ± 0.2 efg	0.9 ± 0.1 ijk	0.8 ± 0.1 f
	8.0	57.3 ± 2.2 hi	14.2 ± 0.6 cd	3.2 ± 0.2 a	0.8 ± 0.1 hij	1.2 ± 0.1 b	59.0 ± 1.7 ghi	16.9 ± 0.7 e	2.4 ± 0.2 cde	0.8 ± 0.2 jk	1.4 ± 0.1 d
$F_{\text{one-way ANOVA}}$		***	***	***	***	***	***		**	***	***

ᶻMean (±SE) separation in columns by Student's *t*, *p*≤0.05

NS, **, ***Nonsignificant or significant at *p*≤0.01, *p*≤0.001, respectively, *n*=48

ᵀNSh=normal shoot

ᵀᵀHSh=hyperhydrated shoot

ᵀᵀᵀHf=hormone free

ʸpercentage of explants producing hyperhydrated shoots only (totally hyperhydrated explants)

ˣshoots from totally hyperhydrated explants not included

Table 3. Effect of cytokinin type and concentration on shoot proliferation from adult-origin explants excised from microshoots produced either on MS medium (I, first culture) or on the same medium as that tested for multiplication (II, subculture)

Cytokinin type	Cytokinin conc (mg·L⁻¹)	I (first culture)					II (subculture)				
		Shooting (%)	Shooting-hyperhydrated shoots only (%)ʸ	Mean NShᵀ number	Mean NSh length (cm)	Mean HShᵀᵀ numberˣ	Shooting (%)	Shooting-hyperhydrated shoots only (%)ʸ	Mean NSh number	Mean NSh length (cm)	Mean HSh numberˣ
Hfᵀᵀᵀ	-	80.2 ± 1.2 c	0.0 ± 0.0 h	1.3 ± 0.1 gh	2.9 ± 0.3 a	0.0 ± 0.0 g	82.0 ± 0.8 e	0.0 ± 0.0 h	1.2 ± 0.1 h	2.9 ± 0.2 a	0.0 ± 0.0 h
ZEA	0.25	83.3 ± 2.3 bc	0.0 ± 0.0 h	1.5 ± 0.2 def	2.1 ± 0.1 bcd	0.0 ± 0.0 g	85.7 ± 2.0 d	0.0 ± 0.0 h	1.6 ± 0.1 efg	2.0 ± 0.4 bc	0.0 ± 0.0 h
	0.5	81.0 ± 2.6 c	0.0 ± 0.0 h	1.4 ± 0.2 efg	1.9 ± 0.2 bcd	0.0 ± 0.0 g	81.0 ± 2.0 d	0.0 ± 0.0 h	1.9 ± 0.2 bcd	1.4 ± 0.1 efg	0.0 ± 0.0 h
	1.0	81.0 ± 1.2 c	0.0 ± 0.0 h	1.5 ± 0.1 def	1.6 ± 0.2 cde	0.0 ± 0.0 g	81.0 ± 2.0 ef	0.0 ± 0.0 h	2.0 ± 0.5 abc	1.5 ± 0.2 def	0.3 ± 0.1 g
	2.0	61.8 ± 1.2 ef	12 ± 0.6 de	1.6 ± 0.1 def	1.4 ± 0.2 def	0.8 ± 0.1 ef	68.3 ± 2.0 g	8.1 ± 0.7 e	2.0 ± 0.5 abc	0.6 ± 0.1 i	1.0 ± 0.1 e
	4.0	54.2 ± 1.7 gh	17 ± 1.0 b	1.7 ± 0.2 cde	0.8 ± 0.1 fg	1.0 ± 0.1 d	54.3 ± 2.0 ij	20.8 ± 1.5 b	2.0 ± 0.4abc	0.5 ± 0.1 i	1.3 ± 1.0 de
	8.0	40.4 ± 2.6 i	28 ± 1.5 a	2.8 ± 0.1 a	0.9 ± 0.1fg	1.8 ± 0.1 b	43.1 ± 1.2 k	30.4 ± 1.4 a	2.5 ± 0.3 a	0.6 ± 0.1 i	2.8 ± 1.0 b
BA	0.25	80.0 ± 2.0 c	0.0 ± 0.0 h	1.2 ± 0.1 h	2.4 ± 0.2 ab	0.0 ± 0.0 g	100.0 ± 0.0 a	0.0 ± 0.0 h	1.5 ± 0.1 fgh	2.6 ± 0.2 ab	0.0 ± 0.0 h
	0.5	83.3 ± 2.6 bc	0.0 ± 0.0 h	1.2 ± 0.1 h	1.6 ± 0.2 cde	0.0 ± 0.0 g	81.0 ± 0.7 ef	0.0 ± 0.0 h	1.7 ± 0.1 efg	2.0 ± 0.3 bc	0.0 ± 0.0 h
	1.0	83.3 ± 2.6 bc	0.0 ± 0.0 h	1.7 ± 0.1 def	1.7 ± 0.2 cde	0.0 ± 0.0 g	81.0 ± 0.9 ef	0.0 ± 0.0 h	2.2 ± 0.3 abc	1.9 ± 0.4 cde	0.8 ± 0.1 f
	2.0	65.8 ± 2.7 ef	11 ± 1.0 ef	2.2 ± 0.3 bc	1.0 ± 0.1 efg	0.8 ± 0.1 ef	68.0 ± 2.1 g	8.0 ± 0.6 d	1.8 ± 0.2 cde	0.8 ± 0.1 hi	1.2 ± 0.1 de
	4.0	61.0 ± 2.2 ef	15 ± 1.0 c	2.0 ± 0.2 cd	1.3 ± 0.1 def	1.2 ± 0.1 cd	55.0 ± 2.0 i	19.0 ± 1.0 b	2.0 ± 0.2 abc	0.7 ± 0.1 i	1.8 ± 0.1 c
	8.0	41.7 ± 1.1 i	29 ± 1.5 a	2.5 ± 0.2 ab	1.0 ± 0.1 efg	2.1 ± 0.1 a	40.9 ± 1.4 k	30.4 ± 2.2 a	2.3 ± 0.3 ab	0.6 ± 0.1 i	3.5 ± 0.1 a
KIN	0.25	97.6 ± 0.9 a	0.0 ± 0.0 h	1.3 ± 0.1 gh	2.0 ± 0.2 bcd	0.0 ± 0.0 g	90.5 ± 0.3 c	0.0 ± 0.0 h	1.4 ± 0.1 fgh	1.7 ± 0.2 cde	0.0 ± 0.0 h
	0.5	100.0 ± 0.0 a	0.0 ± 0.0 h	1.6 ± 0.1 def	1.6 ± 0.2 cde	0.0 ± 0.0 g	90.5 ± 0.7 c	0.0 ± 0.0 h	1.3 ± 0.1 gh	1.8 ± 0.2 cde	0.0 ± 0.0 h
	1.0	100.0 ± 0.0 a	0.0 ± 0.0 h	1.5 ± 0.1 def	1.5 ± 0.2 de	0.0 ± 0.0 g	80.5 ± 2.0 ef	0.0 ± 0.0 h	1.5 ± 0.1 fgh	2.0 ± 0.3 bc	0.0 ± 0.0 h
	2.0	73.0 ± 1.9 d	8 ± 0.7 g	1.7 ± 0.2 cde	1.0 ± 0.1 efg	0.6 ± 0.1 f	70.3 ± 2.1 g	4.7 ± 0.4 f	1.5 ± 0.1 fgh	0.8 ± 0.1 hi	0.4 ± 0.1 g
	4.0	63.7 ± 1.8 ef	10 ± 0.9 f	1.5 ± 0.1 def	0.8 ± 0.1 fg	0.6 ± 0.1 f	60.3 ± 1.6 h	9.9 ± 0.7 cd	1.5 ±0.2 fgh	0.9 ± 0.1 hi	0.7 ± 0.1 f
	8.0	54.2 ± 2.3 gh	13 ± 1.0 d	2.6 ± 0.2 ab	1.0 ± 0.1 efg	0.9 ± 0.1 ef	52.0 ± 1.7 ij	18.4 ± 0.5 b	2.0 ± 0.2 abc	1.0 ± 0.3 fgh	1.1 ± 0.1 e
2IP	0.25	100.0 ± 0.0 a	0.0 ± 0.0 h	1.3 ± 0.1 gh	1.9 ± 0.2 bcd	0.0 ± 0.0 g	95.2 ± 1.2 b	0.0 ± 0.0 h	1.3 ± 0.1 gh	1.9 ± 0.2 cde	0.0 ± 0.0 h
	0.5	87.4 ± 1.9 b	0.0 ± 0.0 h	1.5 ± 0.1 def	1.8 ± 0.2 cde	0.0 ± 0.0 g	90.8 ± 1.5 c	0.0 ± 0.0 h	1.2 ± 0.1 h	1.6 ± 0.1 cde	0.0 ± 0.0 h
	1.0	87.3 ± 1.9 b	0.0 ± 0.0 h	1.6 ± 0.1 def	1.6 ± 0.2 cde	0.0 ± 0.0 g	86.2 ± 1.5 d	0.0 ± 0.0 h	1.4 ± 0.2 fgh	1.6 ± 0.2 cde	0.0 ± 0.0 h
	2.0	67.1 ± 2.2 e	8 ± 0.8 g	1.4 ± 0.1 efg	1.5 ± 0.3 de	0.7 ± 0.1 f	72.2 ± 2.2 f	8.0 ± 0.7 e	1.7 ± 0.2 efg	1.4 ± 0.3 efg	0.5 ± 0.1 g
	4.0	59.5 ± 2.0 fg	12 ± 0.9 de	1.5 ± 0.2 def	1.2 ± 0.1 cf	0.9 ± 0.1 ef	56.4 ± 1.4 hi	9.6 ± 0.8 cd	1.7 ± 0.2 efg	1.1 ± 0.1 fgh	0.8 ± 0.1 f
	8.0	52.3 ± 1.8 h	16 ± 1.3 bc	2.6 ± 0.2 a	0.7 ± 0.1 g	1.1 ± 0.1 d	50.4 ± 1.5 j	17.0 ± 1.0 b	2.3 ± 0.3 ab	0.6 ± 0.1 i	1.4 ± 0.1 de
$F_{\text{one-way ANOVA}}$		***	***	***	***	***	***	***	**	***	***

ᶻMean (±SE) separation in columns by Student's *t*, $p \leq 0.05$

NS, **, ***Non-significant or significant at $p \leq 0.01$, $p \leq 0.001$, respectively, *n*=48

ᵀNSh=normal shoot

ᵀᵀHSh=hyperhydrated shoot

ᵀᵀᵀHf=hormone free

ᵖercentage of explants producing hyperhydrated shoots only (totally hyperhydrated explants)

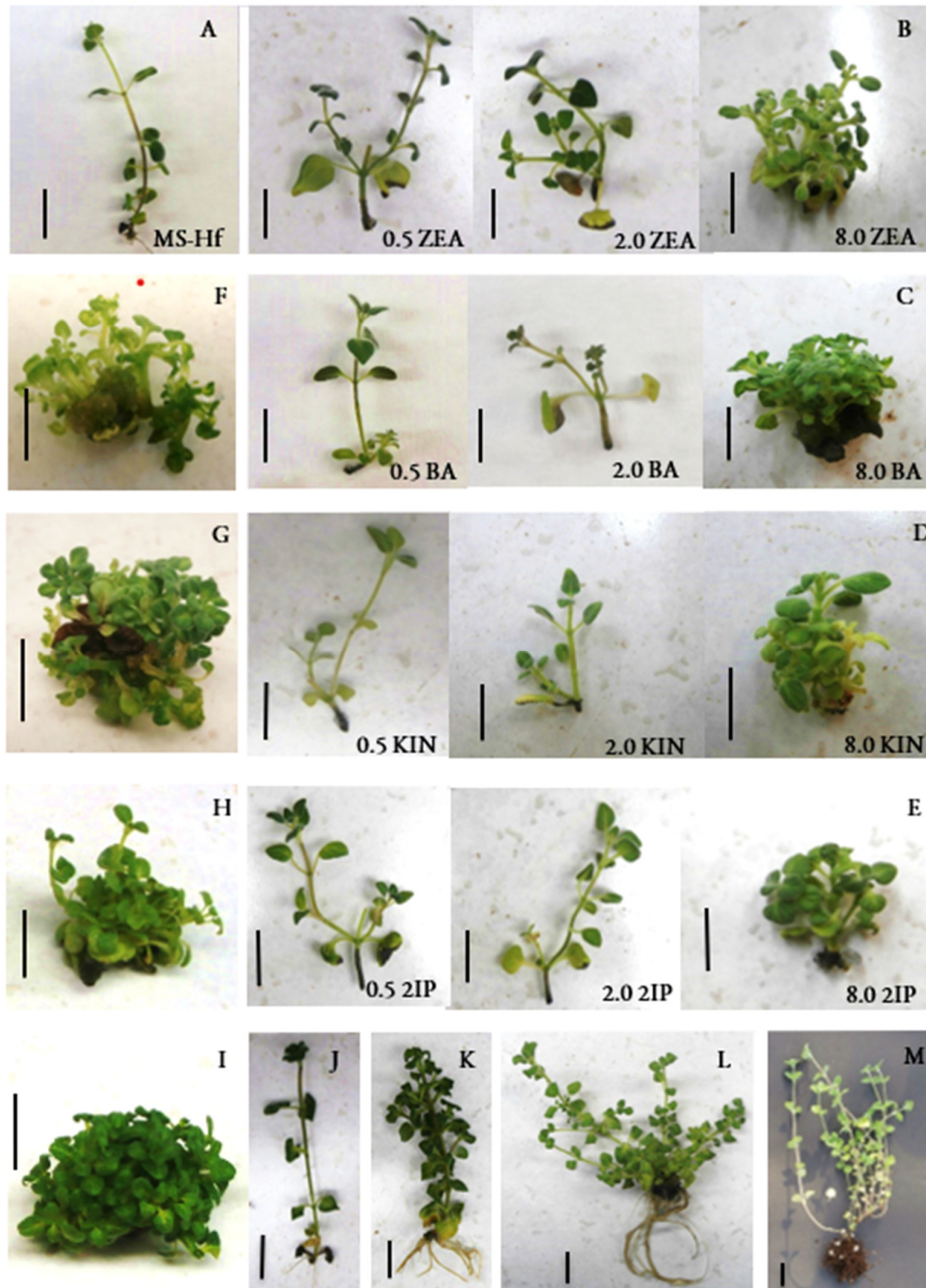ˣshoots from totally hyperhydrated explants not included

Figure 1. Response of *Calamintha cretica* adult-origin nodal explants cultured on MS medium hormone free (Hf) (A), or supplemented with 0.5, 2.0 or 8.0 mg L^{-1} (ZEA) (B), 0.5, 2.0 or 8.0 mg L^{-1} (BA) (C), 0.5, 2.0 or 8.0 mg L^{-1} (KIN) (D), 0.5, 2.0 or 8.0 mg L^{-1} (2IP) (E). Hyperhydric shoots produced on seedling-origin explant at the subculture (II) on MS medium supplemented with 8.0 mg L^{-1} BA (F). Normal and hyperhydric shoots produced on seedling-origin explant on MS medium supplemented with 8.0 mg L^{-1} (G). Normal shoots produced on seedling-origin explant cultured on MS medium supplemented with 8.0 mg L^{-1} BA combined with 0.1 mg L^{-1} NAA (H) or 8.0 mg L^{-1} BA solidified by 12 g L^{-1} agar (I). Microshoot rooted on HF-MS/2 medium (J) or HF-MS/2 medium with 4 mg L^{-1} IBA (K) and shoot cluster (L) rooted on HF-MS/2 medium. Acclimatized plantlet 2.5 months after its *ex vitro* transfer (M). Size bars = 1.0 cm

Effect of cytokinin and auxin combination on shoot proliferation and hyperhydricity

The combination of BA with 0.1 or 0.5 mg L^{-1} NAA into the multiplication medium increased the response of both seedling and adult-origin explants for shoot production and almost eliminated hyperhydricity (Table 4) compared to media supplemented with high BA concentrations only (Table 2). The best response in terms of proliferation occurred when 8 mg L^{-1} BA were combined with 0.1 mg L^{-1} NAA, as a satisfying normal-shoot number and length was induced (Table 4, Figure 1H).

Table 4. Effect of BA and NAA on shoot multiplication from adult- or seedling-origin explants excised from microshoots produced on MS medium

BA/NAA (mg L^{-1})	Adult plant origin explant					Seedling origin explant				
	Shooting (%)	Hyperhydrated shoots (%)	Mean NSh[T] number	Mean NSh length (cm)	Mean HSh[TT] number	Shooting (%)	Hyperhydrated shoots (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number
-/- (Hf[TTT])	93.3 ± 1.6 a[z]	0.0± 0.0 c	1.4 ± 0.1 e	2.3 ± 0.1 a	0.0± 0.0c	100 ± 0.0 a	0.0± 0.0 c	1.4 ± 0.1 e	2.5 ± 0.3 a	0.0± 0.0 c
0.5/0.1	83.3 ± 2.2 bc	0.0± 0.0 c	2.1 ± 0.2 cd	1.1 ± 0.2 bcd	0.0± 0.0 c	100 ± 0.0 a	0.0± 0.0 c	2.3 ± 0.4 bcd	1.8 ± 0.2 cd	0.0± 0.0 c
0.5/0.5	83.3 ± 2.2 bc	0.0± 0.0 c	1.4 ± 0.1 e	1.3 ± 0.1 bc	0.0± 0.0 c	92.2 ± 2.2 b	0.0± 0.0 c	1.8 ± 0.2 de	2.6 ± 0.2 a	0.0± 0.0 c
1.0/0.1	80.0 ± 1.7 bc	0.0± 0.0 c	2.1 ± 0.1 cd	1.0 ± 0.1 cd	0.0± 0.0 c	100 ± 0.0 a	0.0± 0.0 c	2.8 ± 0.4 b	1.7 ± 0.3 cde	0.0± 0.0 c
1.0/0.5	70.2 ± 2.2 d	0.0± 0.0 c	1.9 ± 0.1 de	0.9 ± 0.1 d	0.0± 0.0 c	87.5 ± 2.7 bc	0.0± 0.0 c	2.4 ± 0.3 bcd	1.9 ± 0.3 bcd	0.0± 0.0 c
2.0/0.1	83.3 ± 4.4 bc	7.6 ± 0.4 b	2.0 ± 0.3 d	1.0 ± 0.2 cd	0.3 ± 0.2 b	96.7 ± 1.0 a	6.9 ± 0.3 b	2.6 ± 0.2 bc	1.2 ± 0.1 def	0.4 ± 0.1 b
2.0/0.5	93.3 ± 1.6 a	0.0± 0.0 c	1.8 ± 0.1 de	1.2 ± 0.1 bc	0.0± 0.0 c	96.7 ± 1.0 a	0.0± 0.0 c	2.1 ± 0.2 bcd	0.9 ± 0.1 f	0.0± 0.0 c
4.0/0.1	93.3 ± 1.6 a	8.1 ± 0.4 b	2.0 ± 0.1 d	1.0 ± 0.1 cd	0.3 ± 0.2 b	100 ± 0.0 a	7.0 ± 0.3 b	2.6 ± 0.3 bcd	1.0 ± 0.1 cd	0.4 ± 0.1 b
4.0/0.5	80.0 ± 1.7 bc	0.0± 0.0 c	2.6 ± 0.2 bc	1.4 ± 0.1 b	0.0± 0.0 c	86.7 ± 2.5 c	0.0± 0.0 c	1.9 ± 0.1 cde	0.9 ± 0.1 f	0.0± 0.0 c
8.0/0.1	76.7 ± 2.4 cd	10.8 ± 0.4 a	3.8 ± 0.3 a	1.1 ± 0.1 bcd	0.7 ± 0.6 a	90.0 ± 2.5 bc	10.1 ± 0.4 a	4.5 ± 0.6 a	1.1 ± 0.1 ef	0.6 ± 0.1 a
8.0/0.5	86.7 ± 1.6 ab	0.0± 0.0 c	3.1 ± 0.2 b	1.3 ± 0.1 bc	0.6 ± 0.6 a	97.1 ± 0.8 a	0.0± 0.0 c	2.7 ± 0.3 b	0.9 ± 0.1 f	0.5 ± 0.1 a
$F_{one-way\ ANOVA}$	***	***	***	***	***	***	***	***	***	***

[z]Mean (±SE) separation in columns by Student's *t*, $p \leq 0.05$
NS, ***Nonsignificant or significant at $p \leq 0.001$, respectively, $n=48$
[T]NSh=normal shoot
[TT]HSh=hyperhydrated shoot
[TTT]Hf=hormone free

Effect of agar concentration on hyperhydricity

In a further experiment, it was tested the possibility to reduce hyperhydricity, occurring in the high-BA concentration medium, by increasing the agar concentration. Indeed the increase of agar into the 8 mg L^{-1} BA-medium from 8 to 12 g L^{-1} resulted to very low hyperhydricity and to the highest proliferation, higher than that induced in the so far best for proliferation medium, i.e. that with 8 mg L^{-1} BA and 0.1 mg L^{-1} NAA (Table 5, Figure 1I). The increased agar resulted to both highest explant response rate, similar to that of the Hf-medium, and highest shoot number per explant responded (Table 5). Adult- and seedling origin explants responded in a similar way (Table 5).

The increased agar concentration as a means of treating the hyperhydration was successful for the relative species *C. nepeta* (Vlachou *et al.*, 2019), as well as for the native to the Mediterranean region small shrubby species *Lithodora zahnii* (Papafotiou and Kalantzis, 2009b) and *Globularia alypum* (Bertsouklis *et al.*, 2003), and other herbaceous species (Casanova *et al.*, 2008; Liu *et al.*, 2017; Gerszberg, 2018). The concentration of agar into the medium it seems to regulate the water potential, the diffusion of micronutrients and the uptake of cytokinins by the explants (Pâques and Boxus, 1987; Ziv, 1991b; Debergh *et al.*, 1992).

In vitro rooting and ex vitro acclimatization

Microshoots rooted at 95% on half-strength Hf MS medium (Table 6, Figure 1J). Although, auxins are known to play an important role in root induction through their effect on first cell divisions that lead to root formation (Farooq *et al.*, 2008), microshoots of a number of Mediterranean xerophytes, as *A. barba-jovis*, *Ballota acetabulosa*, *C. nepeta*, *L. monopetalum* (Papafotiou and Martini, 2016; Vlachou *et al.*, 2016a, 2017a, 2017b), have been found to root on full- or half-strength MS medium without an auxin. Half-strength MS medium is quite often used successfully, as in the present study, since rather low nutrient concentrations positively affects rooting (Murashige, 1979; Saha *et al.*, 2011; Vlachou *et al.*, 2017a, 2017b; Zhang *et al.*, 2017). Addition of IBA to the medium did not affect rooting percentage, but it increased root number and reduced root length proportional to the increase of its concentration, while it promoted lateral shoot sprouting (Table 6, Figure 1K). Such negative relation between root number and root length has been shown also in micropropagation of carnation and the Mediterranean species *Teucrium capitatum* and *Limoniastrum monopetalum* (Papafotiou and Martini, 2016; Salehi, 2006; Martini and Papafotiou, 2020). Shoot clusters rooted at 97% on half-strength Hf MS and produced 8.7 roots of 1.1 cm length at average (n=46, Figure 1L).

Rooted microshoots were acclimatized ex *vitro* at 90% and rooted clusters at 100% (Figure 1M), similarly to C. *nepeta* (Vlachou *et al.*, 2016b; 2017b).

Table 5. Effect of agar concentration on shoot multiplication from explants excised from microshoots produced on MS medium and cultured on MS with 8.0 mg L^{-1} BA combined or not with 0.1 mg L^{-1} NAA

Agar conc ($g \cdot L^{-1}$)	BA/NAA conc ($mg \cdot L^{-1}$)	Adult plant origin explant					Seedling origin explant				
		Shooting (%)	Hyperhydrated shoots (%)	Mean NSh[Ŧ] number	Mean NSh length (cm)	Mean HSh[ŦŦ] number	Shooting (%)	Hyperhydrated shoots (%)	Mean NSh number	Mean NS length (cm)	Mean HSh number
8	- (Hf)[ŦŦŦ]	100 ± 0.0 a[z]	0.0 ± 0.0 c	2.7 ± 0.1 d	1.8 ± 0.1 a	0.0 ± 0.0 c	100 ± 0.0 a	0.0 ± 0.0 c	2.9 ± 0.2 c	2.0 ± 0.2 a	0.0 ± 0.0 c
8	8.0/-	63.1 ± 1.7 d	25.3 ± 0.6 a	4.6 ± 0.2 c	0.6 ± 0.1 d	1.8 ± 0.1 a	70.0 ± 1.4 d	19.3 ± 1.1 a	5.2 ± 0.1 b	0.5 ± d	1.2 ± 0.1 a
12	8.0/-	95.2 ± 1.0 b	0.0 ± 0.0 c	11.2 ± 0.4 a	1.0 ± 0.1 c	0.7 ± 0.1 b	92.6 ± 1.4 b	0.0 ± 0.0 c	12.3 ± 0.1 a	0.8 ± 0.2 c	0.7 ± 0.1 b
8	8.0/0.1	86.8 ± 1.4 c	9.0 ± 0.3 b	5.9 ± 0.2 b	1.2 ± 0.1 b	0.9 ± 0.1 b	85 ± 1.4 c	7.4 ± 0.3 b	5.7 ± 0.1 b	1.2 ±b	0.8 ± 0.1 b
$F_{one-way\ ANOVA}$		***	***	***	***	***	***	***	***	***	***

[z]Mean (±SE) separation in columns by Student's *t, p*≤0.05
***significant at *p*≤0.001, *n*=30
[Ŧ]NSh=normal shoot
[ŦŦ]HSh=hyperhydrated shoot
[ŦŦŦ]Hf=hormone free

Table 6. *In vitro* rooting of microshoots as affected by IBA concentration

IBA conc ($m \cdot L^{-1}$)	Rooting (%)	Mean root number per shoot	Mean root length (cm)
-	95 ± 1.4 a[z]	5.2 ± 0.3 b	2.1 ±0.1 a
0.5	95 ±1.4 a	5.3 ± 0.3 b	1.9 ±0.1 b
1.0	88 ±1.8 b	5.7 ± 0.3 ab	1.4 ±0.1 d
2.0	95 ±1.4 a	5.8 ± 0.3 a	1.5 ±0.1 cd
4.0	95 ±1.4 a	5.9 ± 0.2a	1.4 ±0.1 d
$F_{one-way\ ANOVA}$	**	***	***

[z]Mean (±SE) separation in columns by Student's *t, p*≤0.05

Conclusions

C. cretica seeds of recent harvest or up to one-year old germinated at almost 100% and completed their germination in 2 weeks, at 15 to 20 °C, with cardinal temperatures for germination 10 °C and 30 °C. During micropropagation, adult- and seedling-origin single node explants responded in a similar way and exhibited high shoot proliferation (93 to 95% explant response, 11.2 to 12.3 shoots per explant), on MS medium supplemented with 8.0 mg L^{-1} BA and solidified with 12 g L^{-1} agar to prevent hyperhydricity. Almost all microshoots and microshoot clusters rooted on Hf half-strength MS medium and were successfully established at *ex vitro* conditions.

Acknowledgements

Conflict of Interests

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

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