Studies on Seed Germination and Micropropagation of *Clinopodium nepeta*: A Medicinal and Aromatic Plant

Georgia Vlachou, Maria Papafotiou¹, and Konstantinos F. Bertouklis
Laboratory of Floriculture and Landscape Architecture, Department of Crop Science, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece

Additional index words. adult and seedling-origin explants, agar concentration, auxin, *Calamintha nepeta*, cytokinin, hyperhydricity, rooting, seed ecophysiology

Abstract. Seed ecophysiology and micropropagation of *Clinopodium nepeta*, an aromatic Mediterranean plant with pharmaceutical and horticultural uses was investigated. The optimum germination temperature of seeds stored at room temperature for 0, 6, or 12 months was 15 to 20 °C (100% germination completed in 10 to 14 days) and cardinal temperatures were defined at 10 and 30 °C (80% to 82% and 62% to 76% germination, respectively). Six or 12 months of storage did not seem to affect germination, although 12-month-old seeds germinated at higher percentage and completed germination earlier at 15 °C than at 20 °C. Concerning micropropagation, shoot multiplication at subcultures of both adult plant- and seedling-origin nodal explants was tested on Murashige and Skoog (MS) medium supplemented with various cytokinin types, i.e., zeatin (ZEA), 6-benzyladenine (BA), kinetin (KIN), and 6-γ-γ-(dimethylallylamino)-purine (ZIP), at various concentrations from 0.0 to 8.0 mg·L⁻¹. Both explant types presented a rather similar response during in vitro culture. Increasing concentration of all cytokinin types resulted in an increase in shoot number per responding explant (1.1–5.3) and in most cases a decrease in shoot length (0.6–3.4 cm). Increasing cytokinin concentration induced hyperhydricity to a number of shoots (0.1–6.5) per explant, mostly when ZEA and BA were used. Supplementing the MS medium with 8.0 mg·L⁻¹ BA combined with 0.1 mg·L⁻¹ 1-naphthaleneacetic acid (NAA) led to almost elimination of hyperhydricity and very satisfactory shoot production (80%/88% explant response and 6.5/7.5 shoot number per responding explant for seedling- / adult-origin explants, respectively). Alternatively, increasing the agar concentration to 12.0 g·L⁻¹ and supplementing the medium with 8.0 mg·L⁻¹ BA only, resulted in the same effect on eliminating hyperhydricity, such as the addition of NAA, and in the best shoot multiplication response achieved in this study (100% explant response, 9.4/9.9 shoots per explant for seedling-/adult-origin explants, respectively). Microshoots rooted abundantly (92% to 100%) on half-strength MS medium, either Hf or supplemented with 0.5 mg·L⁻¹ to 4.0 mg·L⁻¹ indole-3-butyric acid (IBA). The addition of IBA to the rooting medium, regardless of its concentration, affected only the root length by increasing it 2- to 3-fold. Microshoot clusters produced on multiplication media rooted at 96% when cultured on Hf half-strength MS medium. Rooted microshoots and shoot clusters survived at 80% to 100%, respectively, after ex vitro acclimatization in peat:perlite 1:1 (v/v).

Clinopodium nepeta (L.) Kuntze (f. Lamiales, syn.: *Melissa nepeta* L. and the well-known *Calamintha nepeta* (L.) Savi (lesser calamint), name originated from the Greek word “kalos” meaning beautiful and “minthe” meaning mint (Tenenbaum, 2003), is a strongly aromatic, hemicryptophyte perennial herb that grows at rocky places of western and southern Europe from 0 to 1,500 m above sea level (Filibeck, et al., 2012; Pignatti, 1982). The plant has gray-green, fragrant (between mint and oregano) leaves and it bears inflorescence with small, pale lilac to whitish flowers from early

summer to midautumn (Blamey and Grey-Wilson, 2000).

Since antiquity the species has had various uses (Jashemski, 1999). The leaves, rich in flavonoids, are used in traditional medicine (Bandini and Pacchiani, 1981; Montesano et al., 2012), in Italy and Portugal it is used for food seasoning (Pardo-de-Santayana et al., 2007), and essential oil from leaves could be used as fragrance for insect repellent products (Alan et al., 2011; Božović and Ragno, 2017; Drapeau et al., 2009).

C. nepeta has potential use as an ornamental landscape plant or groundcover for urban and suburban parks, gardens, and green roofs (Caneva et al., 2013; Casalini et al., 2017; Dunnett et al., 2008), as well as in restoration projects of Mediterranean archae-

ological sites (Papafotiou et al., 2017a), and in the creation of meadows with native plant species (Gilbert and Anderson, 1998; Maurer et al., 2000), particularly in soils with low fertility (Bretzel et al., 2009a; Vannucchi et al., 2015). It showed good levels of coverage, great survival, size, and flowering performance on green roofs (Benvenuti and Bacci, 2010; Dunnett et al., 2008), as well as self-sowing and acceptable percentage of emergence (Benvenuti, 2014; Casalini, et al., 2017; Nagase et al., 2013). However, there is no published information on *C. nepeta* optimal and cardinal temperatures for germination, or possible seed dormancy suggested by Bretzel et al. (2009b) to explain the rather low germination (64%) found at their experiments. Casalini et al. (2017) used chilling pretreatment of the seeds but the rate of germination remained moderate.

There is limited published work on clonal propagation of *C. nepeta*. In a preliminary publication, we reported in vitro culture of the species, starting from adult plants, on MS medium supplemented with ZEA or BA, with rather low shoot proliferation (Vlachou et al., 2016b).

The aim of the present work was to develop efficient protocols for seed and clonal propagation of *C. nepeta* to facilitate its introduction in the horticultural and pharmaceutical industry. Seed germination, as well as micropropagation starting with either adult plant- or seedling-origin explants were studied. Micropropagation could facilitate the introduction of suitable clones to the pharmaceutical industry as well as breeding programs. The use of seedlings as stock plant material for micropropagation could lead to a high proliferation rate like in other Mediterranean native species (Papafotiou and Martini, 2016; Papafotiou et al., 2013). Further, the use of seedlings for either in vitro or ex vitro propagation could enhance the higher genetic diversity, which is desirable when native plants are reintroduced in the landscape, or contribute to the selection of particular genotypes of high medicinal value (Sarasan et al., 2011). Specifically, in this study were investigated 1) the effect of storage and temperature on *C. nepeta* seed germination ability, and 2) the effect of various plant growth regulators and agar concentrations on in vitro shoot proliferation, elimination of hyperhydricity problems, and microshoot rooting.

Materials and Methods

Plant material

Wild, adult plants found at Amphiarion Archaeological Site, Oropos, Attiki, Greece (lat. 38°17'28.5"N, long. 23°50'43.5"E) were used in June 2012 for 1) excision of stem cuttings for stock plant establishment in the greenhouse of the Laboratory of Floriculture and Landscape Architecture, Agricultural University of Athens (lat. 37°58'58.0"N, long. 23°42'19.2"E), and 2) excision of shoot tip explants for in vitro culture establishment.

Received for publication 25 Feb. 2019. Accepted for publication 29 May 2019.

¹Corresponding author. E-mail: mpapaf@aau.gr.

Seed germination

For germination studies, seeds were collected, in Aug. 2014, from the greenhouse growing stock plants (see previously). Seeds were left to dry for 2 to 3 d and then stored in the dark at room temperature. Fifteen days after harvest, or after 6 and 12 months of storage, seeds were surface-sterilized with 15% (v/v) commercial bleach (4.6% w/v sodium hypochlorite) containing 1 to 2 drops of Tween 20 (polyxyethylenesorbitan monolaurate, MERCK) for 10 min, rinsed four times (3 min each) with sterile distilled water and put to germinate in 9-cm petri dishes with 20 mL of solid (8 g·L⁻¹ agar), half-strength MS medium (Murashige and Skoog, 1962) at 5, 10, 15, 20, 25, 30, 35, and 40 °C, and 16-h cool white fluorescent light (37.5 μmol·m⁻²·s⁻¹)/8-h dark photoperiod. Germination was defined according to the rules of the International Seed Testing Association (1999), after the appearance of the radicle at least 2 mm long. T₅₀ is defined as time for 50% germination of seeds.

Micropropagation

Seedling-origin explants. Single-node explants were excised from 1-month-old seedlings (2–3 explants from each seedling) grown in vitro. Fifteen days after the completion of germination, seedlings were transplanted from half- to full-strength MS medium to enhance the rapid production of an adequate number of nodes to be used as explants. The seedlings were cultured at 25 °C and 16-h cool white fluorescent light (37.5 μmol·m⁻²·s⁻¹)/8 h dark photoperiod for 30 d.

Adult-origin explants. Single-node explants were excised from microshoots of in vitro cultures grown on hormone-free (Hf) MS medium. The in vitro cultures were initiated from shoot tip explants excised from adult wild plants (see previously) and cultured on MS medium supplemented with 1.0 mg·L⁻¹ BA. Details on this are given in Vlachou et al. (2016b). The cultures were maintained with a number of subcultures on the initiation medium followed by one subculture on Hf-MS medium.

Effect of cytokinin type and concentration on shoot multiplication

Adult plant- or seedling-origin explants were cultured on MS medium supplemented with cytokinin of four types, i.e., ZEA, BA, KIN, and 2IP, at three concentrations, i.e., 0.5, 2.0, and 8.0 mg·L⁻¹, in all possible combinations. Hf-MS medium was also used as control. One subculture followed, whereby single-node explants were subcultured on the same fresh medium as each one of which had originated.

Effect of plant growth regulators and agar concentration on hyperhydration and shoot multiplication

To address hyperhydration problems occurring at cultures on media with high concentrations of cytokinins, 1) the addition of auxin to the proliferation medium and 2) the

increase of agar concentration were tested. The effect of applied treatments on shoot multiplication was also recorded. Single-node explants of adult or seedling origin were cultured on MS medium 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 NAA, in all possible combinations, and Hf-MS medium was used as control. In another experiment, an MS medium solidified with 8.0 g·L⁻¹ agar and supplemented with 8.0 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA was tested for shoot multiplication and hyperhydration vs. MS 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

Based on results of a previous study of ours (Vlachou et al., 2016b), microshoots, 2.0 to 2.5 cm long, were transferred on half-strength MS medium either Hf or supplemented with 0.5, 1.0, 2.0, or 4.0 mg·L⁻¹ IBA. Also, microshoot clusters produced on multiplication media were put on Hf-half-strength MS medium for rooting.

Ex vitro acclimatization and establishment

Rooted plantlets were transferred into 500-mL containers (eight plantlets per container), on a mixture of peat (High-more with adjusted pH up to 5.5 to 6.5; Klasmann-Delmann GmbH, Geeste, Germany) and perlite (particle diameter 1 to 5 mm; Perloflor, ISOCON S.A., Athens, Greece) 1:1 (v/v), covered for 7 d with transparent plastic wrap (SANITAS, Sarantis S.A., Greece), in a growth chamber (20 °C and 16-h cool white fluorescent light 37.5 μmol·m⁻²·s⁻¹/8-h dark photoperiod). After 7 d, they were transferred to a heated greenhouse, in the mist (substrate temperature 22 °C maintained by thermostatically controlled electric heating cable), where they were maintained for 10 d and then transferred on a greenhouse bench for 30 d. Recording of acclimatization was taken at the end of the 30-d period. Following, young plants were transplanted in pots with peat:perlite (2:1, v/v) and fertilized monthly with 2.0 g·L⁻¹ of a complete water-soluble fertilizer (Nutrileaf 60, 20–20–20; Miller Chemical and Fertilizer Corp., Hanover, PA). Four months later, data for ex vitro establishment were recorded.

In vitro culture conditions and data recording

Initial in vitro cultures were established in test tubes (25 × 100 mm, one explant per tube, 10 mL medium) covered with transparent plastic wrap (SANITAS). All other in vitro cultures, including rooting experiments, were established in 100-mL Sigma glass vessels covered by Magenta B-Caps (four explants per vessel, 25 mL medium). The seed germination medium contained 20 g·L⁻¹ sucrose and all the other 30 g·L⁻¹ sucrose. All media were solidified with 8 g·L⁻¹ agar, except in hyperhydration experiments where 12 g·L⁻¹ agar was also used. The medium pH was adjusted to 5.7, before agar and autoclaving

(121 °C, 20 min). All in vitro cultures were maintained at 25 °C 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. In shoot proliferation experiments, data on the percentage of explants that responded producing normal shoots (NS) alone or in combination with hyperhydrated shoots (HS) were recorded; explants that produced only HS were not calculated in the shooting percentage. NS and HS number per responding explant and shoot length were also recorded. In rooting experiments rooting percentage and root number and length were recorded.

The survival rate for ex vitro acclimatization was recorded 40 d after transfer at the greenhouse and data on establishment 4 months later.

Statistical analysis

The completely randomized design was used in all experiments and the significance of the results was tested by one- or two-way analysis of variance. The means of the treatments were compared by the Student's *t* test at *P* ≤ 0.05 (JMP 11.0 software; SAS Institute Inc., Cary, NC). All the experiments were repeated twice with similar results.

Results and Discussion

Germination. *C. nepeta* seeds showed a remarkable germination ability (80% to 100%) and in a short period of time (10–16 d), in the light (16-h photoperiod) and at a wide range of temperatures (i.e., 10 to 25 °C), without any pretreatment; even at 30 °C germination was 66% to 76% (Table 1). The germination percentages of the studied population were higher compared with data in previous studies by Casalini et al. (2017) and Benvenuti and Bacci (2010) who reported germination percentages 50% to 60%. The high germination, without any seed pretreatment, soon after seed harvest and for a year after, indicated that there was no dormancy, similarly to other *Clinopodium* species, as *C. sandaliotica* (Mattana et al., 2016) and *C. vulgare* (Angelova et al., 1994). The Lamiaceae family includes species either nondormant or physiologically dormant (Baskin and Baskin, 1998). Other Lamiaceae species, such as *Origanum dictamnus*, *Salvia pomifera* ssp. *pomifera*, and *S. fruticosa*, germinated also at high percentages at temperatures ranging from 10 to 25 °C (Thanos and Doussi, 1995).

The optimum germination temperature was 15 to 20 °C irrespectively of storage period (Table 1), as shown for other Mediterranean species either of the Lamiaceae family [i.e., *Sideritis syriaca* ssp. *syriaca* (Thanos and Doussi 1995), *Coridothymus capitatus*, *Origanum vulgare* subsp. *hirtum*, *Satureja thymbra* (Thanos et al., 1995)] or others [i.e., *Phlomis brevibracteata*, *Pimpinella cyprica* ssp. *occidentalis* (Kadis and Georghiou 2010) and *Anthyllis barba-jovis* (Morbidoni et al., 2008; Trigka and

Papafotiou, 2017)]. Temperature of 20 °C promoted germination of *Sideritis pungens* and *Spiraea chamaedryfolia* (Estrelles et al., 2010), whereas 15 °C was found to be more effective for germination of the Mediterranean species *Dianthus fruticosus* (Papafotiou and Stragas, 2009), *Globularia alypum* (Bertsouklis and Papafotiou, 2010), and all the *Arbutus* species found in Greece (Bertsouklis and Papafotiou, 2013).

Cardinal germination temperatures were defined at 10 and 30 °C (80% to 82% and 62% to 76% germination, respectively). At

10 °C germination was slightly retarded, whereas at 30 °C it was slightly accelerated (Table 1). Six or 12 months of storage at room temperature did not seem to affect germination, although 12-month-old seeds germinated at higher percentage and completed germination earlier at 15 °C (optimum temperature) than at 20 °C (Table 1).

Therefore, it seems that *C. nepeta* seeds are ready to germinate at high rates after ripening and dispersal on the ground, in autumn, when rainy season starts in the Mediterranean region, even in relatively

low temperatures (10 °C). This adaptation to the season characterizes Mediterranean species (Doussi and Thanos, 2002) and especially Lamiaceae species (Pérez-García et al., 2003), which in this way avoid the adverse for germination high summer temperatures.

Effect of cytokinin type and concentration on in vitro shoot multiplication. *C. nepeta* cultures from adult wild plants were established on MS medium supplemented with 1.0 mg·L⁻¹ BA (70% contamination, 75% explant response for shoot production, 2–3 shoots per responding explant, data presented by Vlachou et al., 2016b), whereas Pistelli et al. (2013) used MS with 0.5 mg·L⁻¹ BA for initial establishment of *C. nepeta*. A considerable number of Mediterranean xerophytes, including species of the Lamiaceae family, cultured on a BA-supplemented MS medium responded better at lower BA concentrations (i.e., 0.25 to 0.5 mg·L⁻¹) compared with higher BA concentrations (1.0 to 4.0 mg·L⁻¹) (Papafotiou et al., 2017b). However, similarly to our findings for *C. nepeta*, MS medium containing a higher concentration of BA (1.0 to 2.0 mg·L⁻¹) was found to have best response in terms of shoot formation from nodal explants at the establishment stage for other Lamiaceae species, too, as *Mentha piperita* and *Ocimum gratissimum* (Saha et al., 2010, 2012).

After a number of subcultures on the initiation medium and a final one on Hf-MS medium, single-node explants (adult-origin explants) were excised to test the effect of cytokinin type and concentration on shoot multiplication. Shoot number per adult-origin responding explant was increased by the increase of cytokinin concentration, in all cytokinin types, both in the first culture and the subculture. To the contrary, shoot length was decreased by the increase of cytokinin concentration but only in the first culture (Table 2, Fig. 1A–E). The percentage of adult-origin explants that produced shoots was varying through the different treatments, and in most cases was higher in the Hf

Table 1. In vitro germination of *Clinopodium nepeta* seeds at temperatures shown, after 0, 6, and 12 months of storage at room temperature.

Storage (mo.)/germination temp (°C)	Germination (%)	T ₅₀ (d)	Time (d) for full germination
0/5	0.0 ± 0.0 d ^z	—	—
0/10	82.0 ± 3.7 b	8	16 a
0/15	100.0 ± 0.0 a	4	12 c
0/20	100.0 ± 0.0 a	4	12 c
0/25	80.0 ± 3.0 b	4	14 b
0/30	70.0 ± 5.3 c	2	10 d
0/35	0.0 ± 0.0 d	—	—
0/40	0.0 ± 0.0 d	—	—
6/5	0.0 ± 0.0 d	—	—
6/10	80.0 ± 3.2 bc	10	16 a
6/15	98.0 ± 2.0 a	4	14 b
6/20	100.0 ± 0.0 a	4	14 b
6/25	82.0 ± 2.0 b	6	14 b
6/30	76.0 ± 2.5 c	2	14 b
6/35	0.0 ± 0.0 d	—	—
6/40	0.0 ± 0.0 d	—	—
12/5	0.0 ± 0.0 e	—	—
12/10	82.0 ± 2.5 c	8	14 b
12/15	100.0 ± 0.0 a	4	10 c
12/20	92.0 ± 2.5 b	4	14 b
12/25	84.0 ± 1.6 c	6	16 a
12/30	66.0 ± 2.7 d	4	10 c
12/35	0.0 ± 0.0 e	—	—
12/40	0.0 ± 0.0 e	—	—
Significance of 2-way analysis of variance			
F _{temperature}	—	—	—
F _{storage}	—	—	—
F _{temperature x storage}	*	***	***

^zMean (± SE) separation in columns by Student's *t* test at *P* ≤ 0.05.

*, ***Significant at *P* ≤ 0.05 or 0.001, respectively. n = 5, 20 seeds/petri dish (total 100 seeds per treatment).

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

Cytokinin type	Cytokinin concn (mg·L ⁻¹)	I (first culture)				II (subculture)			
		Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number	Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number
Hf	—	86 a ^z	1.4 f	2.6 b	0.0	80 a	1.2 f	0.8 d	0.0
ZEA	0.5	77 bc	1.5 f	3.4 a	0.0	70 b	1.6 ef	0.8 d	0.0
	2.0	60 d	2.4 de	1.7 cde	0.8	60 cd	2.2 de	1.9 a	1.3
	8.0	57 d	4.4 a	0.9 g	2.2	53 d	4.3 a	1.0 cd	4.4
BA	0.5	80 abc	3.3 bc	1.9 cde	0.0	75 ab	1.6 ef	0.7 d	0.0
	2.0	60 d	3.0 cd	1.4 def	1.3	63 c	3.0 c	1.1 cd	2.4
	8.0	38 e	4.1 ab	0.8 g	1.9	37 e	4.4 a	1.0 cd	4.5
KIN	0.5	75 bc	1.1 f	1.9 cde	0.0	75 ab	1.2 f	0.6 d	0.0
	2.0	83 ab	2.4 de	1.4 def	0.2	77 a	2.7 cd	1.5 b	0.7
	8.0	77 bc	3.5 bc	1.2 fg	0.5	71 ab	3.6 b	0.9 cd	0.9
2IP	0.5	80 abc	1.8 ef	2.1 c	0.0	73 ab	1.6 ef	0.7 d	0.0
	2.0	83 ab	2.5 de	1.4 def	0.1	77 a	2.6 cd	1.2 bc	0.4
	8.0	77 bc	3.5 bc	1.2 fg	0.4	71 ab	3.0 bc	1.0 cd	0.7
F _{one-way ANOVA}		***	***	***	***	***	***	***	***

^zMean separation in columns by Student's *t*, *P* ≤ 0.05.

***Significant at *P* ≤ 0.001 (n = 30).

NSh = normal shoot; HSh = hyperhydrated shoot; Hf = hormone free; ZEA = zeatin; BA = 6-benzyladenine; KIN = kinetin; 2IP = 6-γ-γ-(dimethylallylamino)-purine; ANOVA = analysis of variance.

Fig. 1. Variation in the response of *Clinopodium nepeta* adult-origin explants cultured on Murashige and Skoog (1962) growth medium (MS) hormone free (Hf) (A), or supplemented with 0.5, 2.0, or 8.0 mg·L⁻¹ zeatin (ZEA) (B); 0.5, 2.0, or 8.0 mg·L⁻¹ 6-benzyladenine (BA) (C); 0.5, 2.0, or 8.0 mg·L⁻¹ kinetin (KIN) (D); 0.5, 2.0, or 8.0 mg·L⁻¹ 6-γ-γ-(dimethylallylamino)-purine (2IP) (E). Hyperhydrated shoots produced on seedling-origin explant at the subculture (II) on MS medium supplemented with 8.0 mg·L⁻¹ ZEA (F). Normal shoots produced on seedling-origin explant at the first culture on MS medium supplemented with 8.0 mg·L⁻¹ ZEA (G), 8.0 mg·L⁻¹ BA (H), 8.0 mg·L⁻¹ BA and solidified by 12 g·L⁻¹ agar, or 8.0 mg·L⁻¹ BA combined with 0.1 mg·L⁻¹ 1-naphthaleneacetic acid (NAA) (I). Microshoot (J) and shoot cluster (K) rooted on Hf-MS/2 medium. Acclimatized plantlet 2.5 months after its ex vitro transfer (L). Size bars = 1.0 cm.

medium compared with the cytokinin media, whereas in the case of ZEA and BA, increasing their concentration in the medium decreased shooting response in both the first culture and the subculture (Table 2).

The increase of cytokinin concentration induced hyperhydricity to a number of shoots per adult-origin explant mostly when ZEA or BA was used and particularly in the subculture (Table 2). Some of the explants produced HS only (Fig. 1F) and they were not calculated in the percentage of responding explants. Thus, this is partly the reason for the reduced shooting percentage recorded in high ZEA and BA concentrations (Table 2).

Hyperhydricity is often observed in tissue cultures of Mediterranean xerophytes (Bertsouklis et al., 2003; Papafiotou and Kalantzis, 2009; Trigka and Papafiotou, 2017; Vlachou et al., 2017a, 2017b) found in the most common media and being a result of nonwounding stress (Kevers et al., 2004; Picoli et al., 2001). Cytokinin-induced hyperhydricity is more or less the most difficult to overcome because this phytohormone is essential for shoot formation (Huang et al., 1998). Similar to the present results, tissue cultures of *Allium sativum* and *Lithodora zahni* showed higher percentage of hyperhydricity when the concentration of BA was

increased (Liu et al., 2017; Papafiotou and Kalantzis, 2009), and in the latter hyperhydricity was eliminated when BA concentration was reduced to half.

Using explants of seedling origin, in vitro cultures were established successfully on Hf-MS medium, the percentage of shooting was very high (95%) and an average of two shoots per explant were formed (3.0-cm length) (data not shown). At the multiplication stage (Table 3), the percentage of the seedling-origin explants that responded forming shoots was in general higher compared with adult-origin explants (Table 2), as it has been shown for other Mediterranean xerophytes, as well (Papafiotou et al., 2017b; Vlachou et al., 2016a). The response of the seedling-origin explants was reduced by the highest (8.0 g·L⁻¹) cytokinin concentration in the first culture and by both 2.0 and 8.0 g·L⁻¹ cytokinin concentration in the subculture. The highest number of shoots per seedling-origin explant was produced in the first culture on media supplemented with BA, followed by ZEA, at the highest concentration (Fig. 1G and H), whereas in the subculture the shoot number per explant did not vary considerably between treatments (Table 3). The longest shoots were produced in the Hf medium and often in media with low cytokinin concentration (Table 3).

Similar to adult-origin explants, seedling-origin explants formed some HS when cultured on media containing higher concentrations of cytokinin, particularly in the subculture and when ZEA or BA was used (Table 3).

As expected, no HS were formed on Hf medium independently of explant origin (Tables 2–4), like in a number of other species including Mediterranean xerophytes (Andrade et al., 1999; Ivanova and Van Staden, 2011; Kadota and Niimi, 2003; Vlachou et al., 2017a). However, this medium could not be suggested for the multiplication stage of *C. nepeta*, as it did not lead to a high shoot proliferation rate due to the low number of shoots produced per explant (Fig. 1A).

Effect of cytokinin and auxin combination on in vitro shoot multiplication and hyperhydricity. Taking into account the high cost of ZEA, at a next step of the research, BA in combination with NAA (at various concentrations) was tested as for the effectiveness on proliferation and suppression of hyperhydration. On both explant types, adult and seedling origin, shooting was high in all BA/NAA combinations (85% to 98%) and shoot number per explant was very satisfactory (7.5 in the first culture, 6.5 in the subculture) in the medium containing 8.0 mg·L⁻¹ BA in combination with 0.1 mg·L⁻¹ NAA (Table 4, Fig. 1I). Hyperhydricity was reduced when BA was combined with NAA compared with media containing cytokinin only (Tables 2–4, Fig. 1I). Some explants produced a few HS but only on media with higher BA concentrations (i.e., 2 to 8 mg·L⁻¹), which were eliminated by the highest NAA concentration (0.5 mg·L⁻¹) used (Table 4).

Effect of agar concentration on hyperhydricity. Increasing agar concentration has been successfully used to eliminate hyperhydrated shoot production in micropropagation of the Mediterranean xerophytes *Lithodora zahnii* (Papafotiou and Kalantzis, 2009) and *Globularia alypum* (Bertsouklis et al., 2003). In the present study, the increase of agar concentration from 8.0 g·L⁻¹ to 12.0 g·L⁻¹ was as effective as the combination of BA with NAA in reducing hyperhydricity (Table 5). Moreover, when the medium was supplemented with 8.0 mg·L⁻¹ BA and no NAA,

the increase of agar resulted in 100% shooting, similarly to the Hf medium, and it increased considerably the number of shoots produced per explant (9.9 to 9.4 for adult- and seedling-explant origin, respectively), which was the best multiplication response so far (Table 5, Fig. 11).

In vitro rooting and ex vitro acclimatization and establishment. It has been reported that *C. nepeta* microshoots rooted at higher percentage on Hf-half-strength MS medium compared with the full-strength medium (Vlachou et al.,

2016b). In the present study, adult- and seedling-origin microshoots produced on various multiplication media, rooted promptly (92% to 100%) on half-strength MS medium either Hf (Fig. 1J) or supplemented with 0.5 to 4.0 mg·L⁻¹ IBA (Table 6). The addition of IBA to the rooting medium, regardless of its concentration, affected the root length by increasing it 2- to 3-fold (Table 6). IBA is widely used in rooting media of various species showing more stability than IAA (indole-3-acetic acid), with no phytotoxicity even in relatively high

Table 3. Effect of cytokinin type and concentration on shoot multiplication 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 concn (mg·L ⁻¹)	I (first culture)				II (subculture)			
		Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number	Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number
Hf	—	92 b ^z	1.8 def	3.1 b	0.0	92 a	1.5 c	3.2 a	0.0
ZEA	0.5	92 b	2.6 cd	1.8 cd	0.0	92 a	2.6 ab	2.3 b	0.0
	2.0	100 a	1.9 def	2.3 bc	0.0	46 ef	2.4 ab	2.3 b	2.1
	8.0	79 d	4.1 b	1.2 d	1.7	29 g	2.1 bc	0.8 d	6.5
BA	0.5	92 b	1.8 def	1.5 cd	0.3	83 b	2.4 ab	1.2 d	0.0
	2.0	96 ab	2.3 cde	1.7 cd	0.3	42 f	2.2 abc	1.1 d	1.2
	8.0	83 c	5.3 a	0.9 de	0.8	50 e	3.1 a	0.8 d	1.8
KIN	0.5	85 c	1.6 def	4.1 a	0.0	83 b	2.1 bc	2.1 bc	0.0
	2.0	85 c	1.4 ef	2.7 bc	0.1	70 d	2.3 abc	1.3 d	0.6
	8.0	82 cd	2.1 def	1.6 cd	0.3	73 cd	2.6 ab	0.9 d	1.2
2IP	0.5	100 a	2.3 cde	2.6 bc	0.0	92 a	2.3 abc	2.8 ab	0.0
	2.0	83 c	1.3 f	2.5 bc	0.1	79 c	2.3 ab	1.5 cd	0.4
	8.0	79 d	2.9 c	0.8 e	0.4	83 b	2.6 ab	0.9 d	0.8
<i>F</i> _{one-way ANOVA}		***	***	***	***	***	**	*	***

^zMean separation in columns by Student's *t*, *P* ≤ 0.05.

*, **, ***Significant at *P* ≤ 0.05, 0.01, or 0.001, respectively (n = 30).

NSh = normal shoot; HSh = hyperhydrated shoot; Hf = hormone free; ZEA = zeatin; BA = 6-benzyladenine; KIN = kinetin; 2IP = 6-γ-γ-(dimethylallylamino)-purine; ANOVA = analysis of variance.

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

BA/NAA concn (mg·L ⁻¹)	adult plant-origin explant				Seedling-origin explant			
	Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number	Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number
-/- (Hf)	98 a ^z	2.5 f	2.6 a	0.0	97 a	2.1 cd	2.0 a	0.0
0.5/0.1	98 a	1.7 g	1.6 b	0.0	97 a	1.7 d	1.9 a	0.0
0.5/0.5	92 ab	2.0 fg	1.5 bc	0.0	86 b	1.8 cd	1.8 ab	0.0
1.0/0.1	95 ab	3.0 e	1.7 b	0.0	89 b	3.3 b	1.9 a	0.0
1.0/0.5	86 b	2.4 f	1.7 b	0.0	86 b	1.8 cd	1.8 ab	0.0
2.0/0.1	88 b	4.0 cd	1.3 cd	0.0	70 c	3.7 b	1.1 c	0.3 a
2.0/0.5	95 ab	4.5 b	1.2 d	0.0	83 cb	3.4 b	1.0 c	0.0
4.0/0.1	88 b	4.1 bc	1.4 cd	0.0	73 c	3.6 b	1.2 bc	0.3 a
4.0/0.5	85 b	3.5 de	1.2 d	0.0	73 c	2.9 bc	1.0 c	0.0
8.0/0.1	88 b	7.5 a	1.2 d	0.5 a	80 cb	6.5 a	1.1 c	0.5 a
8.0/0.5	92 ab	4.6 b	1.4 cd	0.6 a	93 a	3.9 b	1.1 c	0.0
<i>F</i> _{one-way ANOVA}		***	***	NS	***	***	***	NS

^zMean separation in columns by Student's *t*, *P* ≤ 0.05.

NS, ***Nonsignificant or significant at *P* ≤ 0.001, respectively (n = 30).

BA = 6-benzyladenine; NAA = 1-naphthaleneacetic acid; NSh = normal shoot; HSh = hyperhydrated shoot; Hf = hormone free; ANOVA = analysis of variance.

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.

Medium		Adult plant-origin explant				Seedling-origin explant			
Agar concn (g·L ⁻¹)	BA/NAA concn (mg·L ⁻¹)	Shooting (%)	Mean NSh number	Mean NSh length (cm)	Mean HSh number	Shooting (%)	Mean NSh number	Mean NS length (cm)	Mean HSh number
8	-(Hf)	100 a ^z	3.0 d	2.5 a	0.0	100 a	2.9 c	2.0 a	0.0
8	8.0/-	68 c	5.0 c	0.8 b	2.7 a	80 d	4.7 b	0.8 b	1.3 a
12	8.0/-	100 a	9.9 a	0.8 b	0.8 b	100 a	9.4 a	0.8 b	0.7 b
8	8.0/0.1	85 b	7.1 b	0.8 b	0.9 b	80 d	5.7 b	0.9 b	1.0 ab
<i>F</i> _{one-way ANOVA}		***	***	***	***	***	***	***	***

^zMean separation in columns by Student's *t*, *P* ≤ 0.05.

***Significant at *P* ≤ 0.001 (n = 30).

BA = 6-benzyladenine; NAA = 1-naphthaleneacetic acid; NSh = normal shoot; HSh = hyperhydrated shoot; Hf = hormone free; ANOVA = analysis of variance.

Table 6. Effect of IBA concentration on in vitro rooting of microshoots.

IBA concn (m·L ⁻¹)	Rooting (%)	Mean root number per shoot	Mean root length (cm)
—	90 b ²	4.6 a	0.7 b
0.5	92 b	5.0 a	1.7 a
1.0	100 a	5.0 a	1.7 a
2.0	92 b	5.4 a	1.8 a
4.0	92 a	5.1 a	1.7 a
<i>F</i> _{one-way ANOVA}	**	NS	***

²Mean separation in columns by Student's *t*, $P \leq 0.05$.

NS, **, ***Nonsignificant or significant at $P \leq 0.01$ or 0.001, respectively (n = 24).

IBA = indole-3-butyric acid; ANOVA = analysis of variance.

concentrations (George et al., 2008). In vitro rooting is also affected by the concentration of nutrient salts in the medium and, similar to the present study, half-strength MS medium has been often proved more effective than the full-strength medium (Murashige, 1979; Saha et al., 2011; Vlachou et al., 2017a, 2017b; Zhang et al., 2017).

Microshoot clusters produced on multiplication media rooted at 96% (data from 46 clusters) when cultured on Hf-half-strength MS medium (Fig. 1K), and produced 8.8 roots per cluster of 1.2-cm length (data not shown).

Single shoot plantlets survived at 80% and rooted shoot clusters at 100% after ex vitro acclimatization and establishment (Fig. 1L).

In conclusion, up to 1-year-old seeds of *C. nepeta* germinated abundantly and at short period at 15 to 20 °C and cardinal temperatures for germination were defined at 10 °C and 30 °C. Concerning micropropagation, adult- and seedling-origin single-node explants responded in a similar way to in vitro culture, and both exhibited high shoot multiplication on MS medium supplemented either with 8.0 mg·L⁻¹ BA and solidified with 12 g·L⁻¹ agar to prevent hyperhydricity or with 8.0 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA. Microshoots and microshoot clusters rooted abundantly on Hf half-strength MS medium and almost all were successfully established at ex vitro conditions.

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