In Vitro Propagation and NaCl Tolerance of the Multipurpose Medicinal Halophyte *Limoniastrum monopetalum*

Aikaterini N. Martini and Maria Papafiotou

Laboratory of Floriculture and Landscape Architecture, Department of Crop Science, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece

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Abstract. *Limoniastrum monopetalum* is an evergreen perennial shrub native to Mediterranean coastal sands and salt marshes. It has adapted to a variety of environmental stresses and is used in traditional medicine and as an ornamental plant. In the present study, an efficient micropropagation protocol for this species was developed to facilitate the production of selected genotypes and promote its wider use. Research has focused on the effects of various cytokinin types [benzyladenine (BA), zeatin, 6-furfurylaminopurine (kinetin) or 6- γ - γ -dimethylallilpurine (2iP)] and concentrations (0.0–4.0 mg·L⁻¹) and various NaCl concentrations (0.0–20 g·L⁻¹) during all stages of in vitro culture. For in vitro establishment, Murashige and Skoog (MS) medium supplemented with 0.5 mg·L⁻¹ BA and 0.0 or 5.0 g·L⁻¹ NaCl was most appropriate (100% explant response, 3–4 shoots per explant, 2 cm shoot length). The best results for shoot multiplication (100% response, 9 shoots per explant, 0.8–1.0 cm shoot length) were obtained with low (0.5 mg·L⁻¹) BA or relatively high (2.0 mg·L⁻¹) kinetin concentrations in the medium; however, 0.5 mg·L⁻¹ kinetin should be preferred in the case of production of multiple rooted microshoots during one stage. The addition of NaCl at relatively low concentrations (2.5 or 5.0 g·L⁻¹) in a medium supplemented with 0.5 mg·L⁻¹ BA doubled shoot multiplication but did not improve shoot elongation (100% explant response, 16 shoots per explant, 0.8 cm shoot length). For in vitro rooting, half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA was most appropriate (97% rooting, 9.4 roots per microshoot, 1.2 cm root length). Regarding the effects of NaCl on in vitro rooting, microshoots were relatively tolerant to NaCl concentrations up to 10.0 g·L⁻¹. The effects of NaCl depend on the micropropagation stage; they are synergistic during shoot multiplication and tolerant during rooting. However, explants responded satisfactorily in its absence, indicating that NaCl was not necessary as a medium component. Ex vitro acclimatization and establishment of plantlets was 100% successful in a mixture of peat:perlite 1:1 or 2:1 (v/v).

Limoniastrum monopetalum (L.) Boiss (*Statice monopetala* L., Plumbaginaceae) is a small, silvery, blue-green evergreen perennial shrub with much-branched, leafy stems that is native to coastal sands and salt marshes in southern Greece and other Mediterranean countries. It has fleshy leaves, is covered with white scales and bright pink flowers that become violet after drying and are borne in

branched spikes during June to August (Blamey and Grey-Wilson, 1993). It is a valuable multipurpose halophyte used in traditional medicine as an antidiarrheic agent against infectious diseases or parasites that cause painful and bloody diarrhea (Chaieb and Boukhris, 1998), for hair tinting and skin tanning (Ksouri et al., 2012), and as an ornamental plant (Lieth and Mochtchenko, 2002). It is rich in phenolics and antioxidants (Aboul-Enein et al., 2012; Trabelsi et al., 2010, 2012, 2013); therefore, it has been proposed as a novel source of natural antioxidants for human consumption and for agro-food, cosmetic, and pharmaceutical industries (Trabelsi et al., 2010, 2012, 2013). Furthermore, it has high potential to be used as a fodder-producing plant because it is rich in nutritive values (carbohydrates and protein) and has low water requirements (Neves et al., 2007; Zahran and El-Amier, 2013).

Halophytes, due to their adaptation to salinity, are mainly suitable for restoration of disturbed landscapes, control of soil ero-

sion and biodiversity maintenance, and for reduction of energy and water consumption, whereas their potential salinity tolerance could be an important commercial feature for reducing production costs (Cassaniti and Romano, 2011). It is noteworthy that there are ≈ 1 billion hectares of salt-affected land worldwide that may be resource opportunities for halotechnologies, such as halophyte crops and landscape plants, which grow better under high salinities (Yensen, 2008). Halophytes adapt to salinity through complex mechanisms of avoidance, evasion, or adaptation processes and tolerance (Breckle, 2002). *L. monopetalum* adsorbs salts and then secretes them through salt glands found on its leaves, a strategy that makes it a typical halophyte (Akoumianaki-Ioannidou et al., 2015). Several halophytes deal with frequent changes in salinity level and synthesize several bioactive molecules (primary and secondary metabolites) that display potent antioxidant, antimicrobial, anti-inflammatory, and antitumoral activities (Ksouri et al., 2012).

L. monopetalum is adapted to water deficits, high light intensity, and high temperatures (Neves et al., 2008), and it is capable of growing on soil poor in organic matter (Salama, 2007), which could be attributed to various structural and chemical characteristics that provide defense and protection (Akoumianaki-Ioannidou et al., 2015). Its ecological value as a sand accumulator, salt-tolerant, and wind-breaker (Salama, 2007) should not be ignored. It can grow in oil-contaminated soils (El-Bakatoushi, 2011; Hussein and Terry, 2002) and has the potential of phytoremediation of heavy metals from polluted sites (Cambrollé et al., 2013a, 2013b; Manousaki et al., 2014). All these adaptations make *L. monopetalum* an ideal ornamental plant for xeriscaping and landscape architecture in arid and semiarid Mediterranean areas with adverse conditions, such as restoration of quarries or roadsides, green roofs, archaeological sites, and other disturbed areas.

We have successfully propagated *L. monopetalum* by stem-tip cuttings, more effectively during winter and spring (Akoumianaki-Ioannidou et al., 2016). Our preliminary results regarding its micropropagation (Martini and Papafiotou, 2016) indicated that BA at 0.5 mg·L⁻¹ induced the most and longest shoots of rather small length (they did not exceed 0.8 cm).

Biotechnological tools are important to select, multiply, conserve, and genetically enhance the critical genotypes of medicinal plants in particular (Debnath et al., 2006; Tripathi and Tripathi, 2003). Plant tissue culture techniques offer an integrated approach for the production of standardized quality pharmaceuticals through the mass production of consistent plant material with qualitatively and quantitatively uniform chemical constituents. Furthermore, an ever-increasing demand for uniform medicinal plant-based medicines warrants their mass cloning through plant tissue strategies

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M.P. is the corresponding author. E-mail: mpapaf@aua.gr.

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(Chaturvedi et al., 2007; Debnath et al., 2006).

During ex situ conservation of wild plants from salt-affected coastal habitats using tissue culture, it is important to search for a possible specific requirement of NaCl in the cultivation medium (Freipica and Ievinsh, 2010). Therefore, NaCl was a key factor for in vitro propagation of *Salicornia europaea* (Shi et al., 2006) and *Salicornia brachiata* (Joshi et al., 2012), whereas in *Crithmum maritimum* shoot proliferation was gradually reduced at higher concentrations of NaCl, although shoot height was enhanced (Grigoriadou and Maloupa, 2008). Relatively high NaCl tolerance in conditions of tissue culture was also found for three rare and endangered coastal plant species of the Baltic Sea: *Glaux maritima*, *Dianthus arenarius* spp. *Arenarius*, and *Linaria loeselii* (Freipica and Ievinsh, 2010).

The tissue culture technique has been frequently used as a fast, reliable, and cost-effective alternative tool for the selection of salt tolerance (Hasegawa et al., 1994; Rai et al., 2011; Winicov and Bastola, 1997) to develop salt-tolerant variants of crop plants. This is of immense importance to increase crop productivity because biotic and abiotic stresses generally impose a major threat to agriculture (Rai et al., 2011).

In the present study, in vitro propagation of *L. monoptetalum* was further investigated following our previous work (Martini and Papafotiou, 2016), and the NaCl need as the medium component, or tolerance, was examined to facilitate the production of selected genotypes and promote its wider use in pharmaceutical, cosmetic, and food industries as well as in landscape architecture and restoration, particularly of degraded areas with a Mediterranean climate. Various cytokinins (BA, zeatin, kinetin, 2iP) and their effective concentrations were evaluated regarding in vitro blastogenesis and rooting to determine shoot elongation and maintenance of high shoot multiplication rates. Microshoots from advanced subcultures were cultured on medium with various indole-3-butyric acid (IBA) concentrations (Martini and Papafotiou, 2016) to examine the effects of subculture on root induction and development. Moreover, various NaCl concentrations were tested during establishment, shoot multiplication, and rooting to investigate the in vitro response of *L. monoptetalum* when subjected to NaCl and the possible contribution of NaCl to better proliferation and rooting. Finally, various peat and perlite mixtures were examined for ex vitro acclimatization and establishment to develop an efficient micropropagation protocol for commercial use.

Materials and Methods

Effect of cytokinins. Shoot tips that were 0.5 to 0.7 cm long (which actually were short entire microshoots) excised from a culture established from adult *Limoniastrum monoptetalum* plants on solid (8 g·L⁻¹ agar) Mura-

shige and Skoog (MS) (Murashige and Skoog, 1962) medium with 30 g·L⁻¹ sucrose and 1.0 mg·L⁻¹ benzyladenine (BA) (Martini and Papafotiou, 2016) after two subcultures on the same medium were used as explants. They were cultured on MS medium with 30 g·L⁻¹ sucrose, supplemented with various concentrations (0.5, 1.0, 2.0, and 4.0 mg·L⁻¹) of BA, zeatin, 6-furfurylaminopurine (kinetin) or 6-γ-γ-dimethylallilopurine (2iP) as well as on plant growth regulator-free (PGR-free) medium (control) to find the optimum cytokinin type and concentration for shoot multiplication and elongation. A culture was also maintained with successive subcultures on MS medium with 30 g·L⁻¹ sucrose and 0.5 mg·L⁻¹ BA for the production of microshoots for rooting experiments.

During the shoot multiplication experiments, three replications of 10 explants were used for each treatment and data were collected after 40 d of culture. The “multiplication index” of each culture was calculated by multiplying the percentage of explants that produced shoots (divided by 100) by the mean number of shoots per responding explant and by the mean length of produced shoots, and then dividing by 0.6 (the length of each explant used for subculture). The “multiplication index” gave the proliferation potential of each culture by showing the number of explants that could be provided for a subsequent subculture.

Effects of NaCl on establishment and shoot multiplication. To examine the effects of NaCl on the establishment of in vitro cultures, shoot tip explants including the vegetative apical meristem, usually with two visible nodes, that were 2.0 cm long were collected in July 2016 from *L. monoptetalum* (L.) Boiss young plants (≈2 years old) grown in the Botanical Garden of the Laboratory of Floriculture and Landscape Architecture of Agricultural University of Athens (lat. 37°58'53.94"N, long. 23°42'25.01"E), and were cultured on MS medium with 30 g·L⁻¹ sucrose supplemented with 0.5 mg·L⁻¹ BA and increasing concentrations of NaCl (0.0, 5.0, 10.0, 15.0, and 20.0 g·L⁻¹). Explants were surface-sterilized with 25% (v/v) commercial bleach solution (4.5% sodium hypochlorite) for 10 min. Three replications of eight explants were used for each treatment and data were collected after 40 d of culture.

In the first subculture, shoot tip explants (entire microshoots) ≈1.0 cm long excised from initial culture with various concentrations of NaCl (0.0–20.0 g·L⁻¹) were cultured on MS medium with 0.5 mg·L⁻¹ BA and corresponding concentrations of NaCl. In the second subculture, shoot tip explants (entire short microshoots) ≈0.8 cm long grown on medium with 0.5 mg·L⁻¹ BA and 0.0, 5.0, or 10.0 g·L⁻¹ NaCl were cultured on MS medium with 0.5 mg·L⁻¹ BA and corresponding concentrations of NaCl; explants grown on medium with 5.0 g·L⁻¹ NaCl were also cultured on MS medium with 0.5 mg·L⁻¹ BA and lower concentrations of NaCl (1.25 and 2.5 g·L⁻¹). Three replications of 10 explants were used for each treatment and data were col-

lected after 40 d of culture. Two more subcultures on MS medium with 0.5 mg·L⁻¹ BA without the addition of NaCl were performed to produce microshoots for rooting experiments.

In vitro rooting. For rooting, microshoots 0.8 to 1.0 cm long excised from fourth to sixth subcultures of the culture established by Martini and Papafotiou (2016) on MS medium with 0.5 mg·L⁻¹ BA were cultured on half-strength MS medium with 20 g·L⁻¹ sucrose and various (0.0, 0.5, 1.0, 2.0, and 4.0 mg·L⁻¹) concentrations of indole-3-butyric acid (IBA). Three replications of 15 microshoots were used for each treatment. Data were collected after 35 d of culture.

Effects of NaCl on in vitro rooting. To investigate the effects of NaCl on in vitro rooting, the concentration of NaCl both during the stage of microshoot production and during the root induction stage were considered. The following rooting experiments were performed:

- Microshoots, 1.0 to 1.2 cm long, grown on MS medium with 0.5 mg·L⁻¹ BA and 0.0, 5.0, 10.0, 15.0, or 20.0 g·L⁻¹ NaCl (first subculture) were cultured for rooting on half-strength MS medium with 20 g·L⁻¹ sucrose supplemented with 1.0 mg·L⁻¹ IBA and corresponding concentrations of NaCl or without NaCl.
- Microshoots, 1.0 to 1.2 cm long, grown on MS medium with 0.5 mg·L⁻¹ BA and 0.0, 1.25, 2.5, 5.0, or 10.0 g·L⁻¹ NaCl (second subculture) were cultured for rooting on half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA and corresponding concentrations of NaCl or without NaCl.
- Microshoots, ≈1.0 cm long, grown on MS medium with 0.5 mg·L⁻¹ BA without NaCl (fourth subculture) were cultured for rooting on half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA and 0.0, 1.25, 2.5, 5.0, 10.0, 15.0, or 20.0 g·L⁻¹ NaCl.

Three replications of 10 microshoots were used for each treatment. Data were collected after 35 d of culture.

In vitro culture conditions. All culture media were solidified with 8 g·L⁻¹ agar, and their pH was adjusted to 5.7 before addition of the agar and autoclaving at 121 °C for 20 min. Initial culture occurred in test tubes (25 × 100 mm) with 10 mL medium while covered with plastic wrap (Sanitas; Sarantis S.A., Athens, Greece); subculture and rooting occurred in 145-mL glass vessels with 25 mL medium (four explants and five microshoots per vessel, respectively) while covered with a magenta plastic cup. 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.

Ex vitro acclimatization and establishment. For ex vitro acclimatization, plantlets produced through the typical rooting process (without the addition of NaCl) were transferred to 500-mL trays (eight plantlets per tray) with three different peat (Highmoor with adjusted pH up to 5.5 to 6.5; Klasmann-Deilmann GmbH, Geeste, Germany) and

perlite (particle diameter 1–5 mm; Perloflor, ISOCON S.A., Athens, Greece) mixtures of 0:1, 1:1, or 2:1 (v/v). The trays were covered with transparent plastic wrap (Sanitas) and placed in a growth chamber (25 °C, 16-h photoperiod at 37.5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by cool white fluorescent lamps) for 1 week before their transfer to a heated greenhouse for an additional 7 weeks.

Four replications of eight rooted microshoots were used for each experiment, and their survival and growth were estimated after 60 d of acclimatization. The acclimatized plantlets were then placed separately in plastic square plug trays (cell dimensions: 5.0 $\times$ 5.0 $\times$ 5.0 cm) containing a peat:perlite (2:1, v/v) mixture; 1 month later, they were transplanted to plastic pots (1.3 L) containing the same mixture. They were fertilized every 45 d with 4 g $\cdot$ L⁻¹ of a complete water-soluble fertilizer (Nutrileaf 60, 20–20–20; Miller Chemical and Fertilizer Corp., Hanover, PA) and 100 mL of solution per plant. After 4 months of establishment, plant growth was estimated.

Statistical analysis. The completely randomized design was used for all experiments. The significance of the results was tested by one- or two-way analysis of variance. Treatment means were compared using Student's *t* test at $P \leq 0.05$ (JMP 11.0 software; SAS Institute Inc., Cary, NC). Data regarding percentages were statistically analyzed after arcsine transformation. The SEM of each treatment was calculated.

Results

Effects of cytokinins. Regarding the effectiveness of the cytokinin type and concentration on shoot multiplication, the shooting percentage was more than 93% in all treatments, except when the two highest concentrations of BA, 2.0 mg $\cdot$ L⁻¹ and 4.0 mg $\cdot$ L⁻¹, were used and shooting was slightly reduced (Table 1). More shoots were produced by explants cultured on kinetin medium independently of concentration and on BA medium at low concentrations (0.5 or 1.0 mg $\cdot$ L⁻¹). The most shoots per explant were formed on a medium with 2.0 mg $\cdot$ L⁻¹ kinetin or 0.5 mg $\cdot$ L⁻¹ BA (Table 1, Fig. 1A and C). Microshoots had a rather restricted elongation apart from those produced on 2iP media and PGR-free media (Table 1, Fig. 1A–D), and they were becoming increasingly malformed by increasing the BA concentration from 1.0 to 4.0 mg $\cdot$ L⁻¹ (Fig. 1A). The multiplication index of *L. monoptalum* explants was highest when they were cultured on medium with 1.0 or 2.0 mg $\cdot$ L⁻¹ kinetin or with 0.5 mg $\cdot$ L⁻¹ BA (Table 1).

Apart from shoots, explants formed roots, except those treated with BA at all concentrations and those treated with zeatin or kinetin at the highest (4.0 mg $\cdot$ L⁻¹) concentration (Table 1, Fig. 1A–D). Explants rooted at high percentages in PGR-free medium, but rooting percentages observed in media containing 2iP at all concentrations tested or zeatin or kinetin at 0.5 mg $\cdot$ L⁻¹ were even higher (Table 1).

Effects of NaCl on establishment and shoot multiplication. During establishment on media supplemented with NaCl, almost all explants responded, forming similar number of shoots independently of NaCl concentration. Both shoot length and the multiplication index gradually decreased as the concentration of NaCl was increased (Table 2, Fig. 2A).

During the first subculture on the same type of media, more shoots were produced and a higher multiplication index was recorded for explants cultured with 5 g $\cdot$ L⁻¹ NaCl, followed by those cultured with 10 g $\cdot$ L⁻¹ NaCl, than for those cultured without NaCl. The lowest shoot number and multiplication index were recorded for explants cultured with the highest concentrations of NaCl (15 or 20 g $\cdot$ L⁻¹).

During the second subculture, in which two lower concentrations of NaCl (1.25 and 2.5 g $\cdot$ L⁻¹) were tested, the most shoots per explant and the highest multiplication index were observed in the media that contained 2.5 and 5.0 g $\cdot$ L⁻¹ NaCl. No differences were found in shoot length (Table 2, Fig. 2C).

In vitro rooting. Microshoots cultured on half-strength MS medium supplemented or not with IBA rooted at high percentages regardless of treatment (Table 3). The increase in IBA concentration in the medium led to an analogous increase of the callusing percentage and the quantity of callus formed at the microshoot base; however, no callusing occurred on IBA-free medium (Table 3, Fig. 1E). The increase in the IBA concentration in the rooting medium induced an analogous increase in the root number produced per explant and decrease in root length (Table 3, Fig. 1E).

Effects of NaCl on in vitro rooting. Microshoots excised from the first subculture on multiplication medium with 5.0 or 10.0 g $\cdot$ L⁻¹ NaCl rooted at higher percentages when rooting occurred on medium with corresponding concentrations of NaCl than when NaCl was not used. These were comparable to those that had grown and rooted on medium without NaCl as well as those that had grown on medium with 15.0 g $\cdot$ L⁻¹ NaCl and rooted on medium with or without corresponding concentrations of NaCl or without it, whereas microshoots grown and rooted on medium with 20.0 g $\cdot$ L⁻¹ NaCl presented reduced rooting percentages (Table 4, Fig. 2D). The number of produced roots was highest in microshoots that had grown and rooted in the absence of NaCl and lowest in microshoots that were grown and rooted on medium supplemented with 20.0 g $\cdot$ L⁻¹ NaCl; however, the opposite was observed with root length, which was largest in microshoots grown and rooted on medium supplemented with 20.0 g $\cdot$ L⁻¹ NaCl and smallest in microshoots grown and rooted on medium without NaCl or supplemented with 5.0 or 15.0 g $\cdot$ L⁻¹ NaCl (Table 4, Fig. 2D).

Regarding microshoots excised from the second subculture, those that had grown on multiplication medium with 2.5–10.0 g $\cdot$ L⁻¹ NaCl and placed for rooting induction on

medium with corresponding concentrations of NaCl rooted at higher percentages (90% to 97%) compared with microshoots grown on the same media, whose rooting occurred on medium without NaCl (63% to 77%) and compared with those that had grown and rooted on medium without NaCl (77%). Microshoots grown on medium with 1.25 g $\cdot$ L⁻¹ NaCl rooted at high percentages (90% to 97%) regardless of whether rooting occurred on medium with or without corresponding concentrations of NaCl (data not shown).

Regarding microshoots grown during the fourth subculture in the absence of NaCl, those that were cultured on rooting medium supplemented with 1.25 to 10.0 g $\cdot$ L⁻¹ NaCl as well as those on the control (without NaCl) rooted at equally high percentages. However, the rooting percentages of microshoots cultured on rooting medium supplemented with higher concentrations of NaCl (15.0–20.0 g $\cdot$ L⁻¹) were gradually reduced as the NaCl concentration was increased (Table 5, Fig. 2E). The most roots per microshoot were produced in the control, whereas both root number and length were significantly reduced when more than 10 g $\cdot$ L⁻¹ NaCl was added to the rooting medium (Table 5, Fig. 2E). At the two highest NaCl concentrations, survival percentages of microshoots were also reduced (Table 5).

Ex vitro acclimatization and establishment. All plantlets transplanted to three different peat:perlite mixtures survived after 8 weeks of acclimatization (Table 6). In all mixtures tested, similar numbers of shoots were formed per plantlet; however, shoots were more elongated in mixtures of peat:perlite of 1:1 and 2:1 (v/v) compared with plain perlite (Table 6, Fig. 1F). Plantlets grown on plain perlite had reddish leaves as an indication of stress, probably due to water issues. More and longer roots were formed in both mixtures of peat:perlite compared with plain perlite (Table 6, Fig. 1F). Acclimatized plants transplanted to peat:perlite 2:1 (v/v) mixture for growth and fertilized every 45 d with 4 g/L Nutrileaf 20–20–20 were all successfully established after 4 months, forming $\approx$ 1.7 shoots/plantlet that were 19.4 cm long (Fig. 1G).

Discussion

L. monoptalum shoot tip explants produced shoots, even without the presence of a cytokinin in the culture medium. However, all cytokinin types at all concentrations tested, apart from zeatin and 2iP at the lowest concentration (0.5 mg $\cdot$ L⁻¹), considerably increased the number of shoots produced per explant; the shoot number was greatest on medium containing 2.0 mg $\cdot$ L⁻¹ kinetin or 0.5 mg $\cdot$ L⁻¹ BA. Longer shoots were produced on 2iP media and PGR-free media, but fewer shoots per explant were formed; on other media, the microshoot length was rather restricted. Similarly, the best results for shoot multiplication of *Limonium thiniense* (Lledó et al., 1996) and *Limonium cavanillesii* (Amo-Marco and Ibañez, 1998) were

Table 1. Effects of cytokinin type and concentration (mg·L⁻¹) on the response of *L. monopetalum* explants at the multiplication stage.

Cytokinin		Shooting (%)	Mean shoot number	Mean shoot length (cm)	Mult. index ^z	Rooting (%)
Type	Concn					
Control ^y	0.0	93.3 ± 4.2 b ^x	1.3 ± 0.1 i	1.9 ± 0.1 b	3.8 ijk	76.7 ± 13.1 c
BA	0.5	100.0 ± 0.0 a	8.5 ± 0.5 a	0.8 ± 0.0 g	11.8 bc	0.0 ± 0.0 g
	1.0	93.3 ± 4.2 b	5.7 ± 0.5 c	0.5 ± 0.0 h	4.7 ghi	0.0 ± 0.0 g
	2.0	86.7 ± 4.2 c	3.9 ± 0.4 de	0.5 ± 0.0 h	3.1 jk	0.0 ± 0.0 g
	4.0	80.0 ± 0.0 d	2.3 ± 0.3 fgh	0.6 ± 0.0 h	1.9 k	0.0 ± 0.0 g
Zeatin	0.5	93.3 ± 4.2 b	2.2 ± 0.2 ghi	1.4 ± 0.1 c	4.6 hij	80.0 ± 7.3 bc
	1.0	100.0 ± 0.0 a	2.2 ± 0.2 gh	1.2 ± 0.0 cd	4.6 hij	43.3 ± 9.5 ef
	2.0	100.0 ± 0.0 a	3.3 ± 0.3 def	1.1 ± 0.0 de	6.1 gh	23.3 ± 10.9 fg
	4.0	100.0 ± 0.0 a	4.0 ± 0.3 d	1.0 ± 0.0 efg	6.7 efg	0.0 ± 0.0 g
Kinetin	0.5	100.0 ± 0.0 a	5.0 ± 0.3 c	1.2 ± 0.0 cd	10.2 cd	90.0 ± 4.5 bc
	1.0	100.0 ± 0.0 a	6.7 ± 0.6 b	1.1 ± 0.0 de	12.6 b	60.0 ± 5.2 d
	2.0	100.0 ± 0.0 a	9.1 ± 0.5 a	1.0 ± 0.0 ef	15.5 a	43.3 ± 6.1 ef
	4.0	100.0 ± 0.0 a	5.5 ± 0.4 c	0.8 ± 0.0 fg	7.7 ef	0.0 ± 0.0 g
2iP	0.5	100.0 ± 0.0 a	1.6 ± 0.1 hi	2.3 ± 0.1 a	6.2 fgh	100.0 ± 0.0 a
	1.0	100.0 ± 0.0 a	1.9 ± 0.1 hi	2.4 ± 0.1 a	7.7 ef	100.0 ± 0.0 a
	2.0	100.0 ± 0.0 a	2.8 ± 0.3 fg	2.4 ± 0.1 a	11.2 bc	86.7 ± 6.7 bc
	4.0	100.0 ± 0.0 a	3.0 ± 0.2 efg	1.7 ± 0.1 b	8.7 de	93.3 ± 4.2 ab
Significance						
<i>F</i> _{interaction}		**	**	**	**	**
<i>F</i> _{one-way ANOVA}		**	**	**	**	**

^zMultiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6.

^yExcluded from two-way analysis of variance.

^xMean values (n = 30) (±SE) in each column followed by the same lowercase letter do not differ significantly at *P* ≤ 0.05 according to Student's *t* test.

**Significant at *P* ≤ 0.01.

BA = benzyladenine; kinetin = 6-furfurylamino-purine; 2iP = 6-γ-γ-dimethylallilopurine.

Fig. 1. Characteristic shoot growth and multiplication of explants subcultured on a medium supplemented with benzyladenine (BA) (A) or zeatin (B) or kinetin (C) or 2iP (D) at marked concentrations (mg·L⁻¹). Typical rooting of microshoots cultured on half-strength Murashige and Skoog (MS) medium supplemented with marked indole-3-butyric acid (IBA) concentrations (mg·L⁻¹) (E), growth of plantlets transplanted on marked mixtures (v/v), 8 weeks after ex vitro acclimatization (F), and 6-month-old ex vitro established plantlets (G). Size bars = 1.0 cm.

obtained with high (1.0–5.0 mg·L⁻¹) kinetin or low (0.1–0.5 mg·L⁻¹) BA concentrations. For *Plumbago zeylanica*, BA was more effective than kinetin and the best shoot proliferation was achieved by rather low (0.5–1.5 mg·L⁻¹) BA concentrations

(Lubaina et al., 2011). The small size of the produced microshoots has been pointed out in *Limonium cavanillesii*; shoots were elongated only when cultured on a medium supplemented with a high concentration (5.0 mg·L⁻¹) of kinetin or 2iP, whereas an increase

in the BAP concentration (up to 5.0 mg·L⁻¹) increased the tendency for abnormalities in shoot development (Amo-Marco and Ibañez, 1998). For *Crithmum maritimum*, compared with the control, the shoot height was reduced by the addition of BA; however, 3.3 mg·L⁻¹ BA produced microshoots that were malformed, succulent, and chlorotic, with initial hyperhydricity symptoms (Grigoriadou and Maloupa, 2008). Similarly, during our work, *L. monopetalum* microshoots were becoming increasingly malformed by increasing the BA concentration from 1.0 to 4.0 mg·L⁻¹.

Simultaneously with shooting, explants rooted at high percentages on plant growth regulator-free media and 2iP media, as well as on 0.5 mg·L⁻¹ zeatin media or kinetin media, indicating that low cytokinin strength was indifferent to or even promoted rooting, whereas BA totally suppressed rooting. During the establishment stage, roots were also formed at high percentages in the medium without plant growth regulators and in medium with 1.0 mg·L⁻¹ zeatin, whereas BA at 1.0 mg·L⁻¹ suppressed rooting (Martini and Papafotiou, 2016). Similarly, rooting was observed during the shoot multiplication stage of *Crithmum maritimum* on media containing 0.0 to 3.3 mg·L⁻¹ BA, and the rooting capacity of explants was decreased gradually by increasing the BA concentration, especially at concentrations higher than 1.1 mg·L⁻¹ (Grigoriadou and Maloupa, 2008). BA was found to inhibit rooting more than zeatin and kinetin in *Lens culinaris* (Fratini and Ruiz, 2002). However, BA at 1.0 mg·L⁻¹ combined with 0.5 mg·L⁻¹ IAA was reported to promote root induction of *P. zeylanica* microshoots (Chinnamadasamy et al., 2010), whereas the combination of 0.6 mg·L⁻¹ BA and 0.2 to 0.5 mg·L⁻¹ α-Naphthaleneacetic acid (NAA) had a positive influence on simultaneous proliferation and

Table 2. Effects of NaCl on the response of *L. monopetalum* explants during the establishment stage and the first two subcultures on MS medium with 0.5 mg·L⁻¹ BA.

Stage	NaCl (g·L ⁻¹)	Shooting (%)	Mean shoot number	Mean shoot length (cm)	Multiplication index ²
Establishment	0.0	100.0 ± 0.0 a ^y	3.8 ± 0.4 a	2.2 ± 0.1 a	13.0 ± 1.2 a
	5.0	100.0 ± 0.0 a	2.9 ± 0.4 a	2.0 ± 0.1 a	10.0 ± 1.5 b
	10.0	100.0 ± 0.0 a	3.0 ± 0.3 a	1.7 ± 0.1 b	8.3 ± 0.8 bc
	15.0	100.0 ± 0.0 a	2.7 ± 0.2 a	1.3 ± 0.1 c	5.7 ± 0.6 cd
	20.0	95.8 ± 4.2 a	2.7 ± 0.2 a	1.0 ± 0.1 d	4.3 ± 0.4 d
	Significance	NS	NS	**	**
First subculture	0.0	100.0 ± 0.0 a	8.0 ± 0.6 c	0.7 ± 0.0 d	9.5 ± 0.7 c
	5.0	100.0 ± 0.0 a	12.9 ± 0.8 a	0.9 ± 0.0 bc	18.2 ± 1.1 a
	10.0	100.0 ± 0.0 a	10.2 ± 0.7 b	0.8 ± 0.0 cd	13.0 ± 0.9 b
	15.0	100.0 ± 0.0 a	4.1 ± 0.5 d	1.0 ± 0.1 a	6.2 ± 0.6 d
	20.0	100.0 ± 0.0 a	2.4 ± 0.3 e	1.0 ± 0.1 ab	3.4 ± 0.4 e
	Significance	NS	**	**	**
Second subculture	0.0	100.0 ± 0.0 a	10.3 ± 0.5 bc	0.76 ± 0.02 a	13.1 ± 0.8 bc
	1.25	100.0 ± 0.0 a	8.3 ± 0.6 c	0.81 ± 0.03 a	11.4 ± 1.1 c
	2.5	100.0 ± 0.0 a	16.0 ± 1.0 a	0.84 ± 0.03 a	22.6 ± 1.7 a
	5.0	100.0 ± 0.0 a	15.7 ± 1.0 a	0.83 ± 0.02 a	22.2 ± 1.6 a
	10.0	100.0 ± 0.0 a	11.1 ± 0.8 b	0.81 ± 0.03 a	15.3 ± 1.4 b
	Significance	NS	**	NS	**

²Multiplication index = shooting (%) × mean shoot number per explant × mean shoot length/0.6.

^yMean values (establishment: n = 24; subcultures: n = 30) (±SE) in each column and phase followed by the same lowercase letter do not differ significantly at *P* ≤ 0.05 according to Student's *t* test.

NS, **Nonsignificant at *P* ≤ 0.05 or significant at *P* ≤ 0.01, respectively.

BA = benzyladenine; MS = Murashige and Skoog.

Fig. 2. Typical shooting of *L. monopetalum* shoot-tip explants cultured initially on Murashige and Skoog (MS) medium supplemented with 0.5 mg·L⁻¹ benzyladenine (BA) and marked NaCl concentrations (g·L⁻¹) (A). Characteristic shoot multiplication and growth on explants subcultured on a medium supplemented with 0.5 mg·L⁻¹ BA and marked NaCl concentrations (g·L⁻¹) during the first (B) and second (C) subcultures. Typical rooting of microshoots grown on MS medium supplemented with 0.5 mg·L⁻¹ BA and marked NaCl concentrations (g·L⁻¹) (D) or without NaCl (E) and cultured for rooting on half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA and marked NaCl concentrations (g·L⁻¹). Size bars = 1.0 cm.

rooting of *Crithmum maritimum*, resulting in high rooting percentages and increased numbers of roots (Grigoriadou and Maloupa, 2008). According to De Klerk et al. (2001), cytokinins may strongly inhibit rooting, but certain cytokinins, at low concentrations, may enhance rooting.

On media with 0.5 mg·L⁻¹ kinetin or 2.0 or 4.0 mg·L⁻¹ 2iP, rooted shoot clusters were produced and high shoot numbers per explant and rooting percentages were simultaneously promoted. These media containing cytokinin instead of auxin (as the conventional rooting hormone) have the advantage of producing

rooted clusters of shoots and not a single rooted microshoot; therefore, they could possibly produce more branched plants after ex vitro establishment. Moreover, the attainment of shoot multiplication and rooting during one stage is particularly important in commercial tissue culture because labor is the highest cost during micropropagation, corresponding to more than 65% of total costs; therefore, it is desirable to minimize the number of transfers during in vitro culture and general handling of the plant material (Nianiou-Obeidat and Iconomou-Petrovich, 1998). Microshoots rooted at equally high percentages at IBA concentrations from 0.0 to 4.0 mg·L⁻¹, in agreement with reports of *L. thiniense* (Lledó et al., 1996) and *L. cavanillesii* (Amo-Marco and Ibañez, 1998) but in contrast to our initial results that showed higher rooting percentages in rooting media supplemented with IBA compared with the hormone-free medium (Martini and Papaftiou, 2016). However, this discordance in our results could be attributed to the improvement of rooting capacity of microshoots as subcultures progressed, which has been reported for another Mediterranean xerophyte *Teucrium capitatum* L. (Papafotiou and Martini, 2016) as well as for various fruit trees (Al-Maari et al., 1994; Hou et al., 2010; Noiton et al., 1992). The increase in the IBA concentration in the medium led to an analogous increase in callus formation at the base of microshoots, as observed in *P. zeylanica* (Sahoo and Debata, 1998; Selvakumar et al., 2001). High in vitro rooting percentages after culture on medium supplemented with 0.1 to 1.5 mg·L⁻¹ IBA or IAA have also been recorded for other plants of the same family, such as *Plumbago* sp. (Bhadra et al., 2009; Lubaina et al., 2011) and *Limonium* sp. (Amo-Marco and Ibañez, 1998; Huang et al., 2000; Lledó et al., 1996). Although *L. monopetalum* microshoots rooted spontaneously in the

Table 3. Effects of IBA concentration on rooting of *L. monopetalum* microshoots.

IBA (mg·L ⁻¹)	Callusing (%)	Rooting (%)	Mean root number	Mean root length (cm)
0.0	0.0 ± 0.0 d ²	87.9 ± 6.2 a	2.0 ± 0.2 d	3.6 ± 0.3 a
0.5	45.7 ± 7.2 c	94.3 ± 3.7 a	6.2 ± 0.7 c	1.5 ± 0.2 b
1.0	77.1 ± 6.8 b	97.1 ± 2.9 a	9.4 ± 0.6 b	1.2 ± 0.1 bc
2.0	97.1 ± 2.9 a	85.7 ± 8.4 a	13.2 ± 1.1 a	0.9 ± 0.1 c
4.0	100.0 ± 0.0 a	85.0 ± 3.3 a	13.8 ± 1.4 a	0.8 ± 0.1 c
Significance	**	NS	**	**

²Mean values (n = 45) (±SE) in each column followed by the same lowercase letter do not differ significantly at $P \leq 0.05$ according to Student's *t* test.

NS, **Nonsignificant at $P \leq 0.05$ or significant at $P \leq 0.01$, respectively.

IBA = indole-3-butyric acid.

Table 4. Effects of NaCl on rooting of *L. monopetalum* microshoots, which were excised from the first subculture on MS medium supplemented with 0.5 mg·L⁻¹ BA (medium of microshoot origin) and cultured on half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA (rooting medium), both media supplemented with NaCl at concentrations shown.

Microshoot origin medium	Rooting medium			
NaCl (g·L ⁻¹)	NaCl (g·L ⁻¹)	Rooting (%)	Mean root number	Mean root length (cm)
0.0	0.0	96.7 ± 3.3 ab ²	8.0 ± 0.6 a	1.1 ± 0.1 e
5.0	0.0	56.7 ± 6.7 d	5.4 ± 0.6 bcd	2.3 ± 0.3 ab
	5.0	96.7 ± 3.3 ab	5.5 ± 0.5 bc	1.2 ± 0.1 e
10.0	0.0	70.0 ± 9.7 cd	6.5 ± 0.6 b	1.8 ± 0.2 cd
	10.0	100.0 ± 0.0 a	4.1 ± 0.5 de	1.9 ± 0.2 bc
15.0	0.0	91.7 ± 8.3 ab	5.9 ± 0.6 bc	1.4 ± 0.1 de
	15.0	100.0 ± 0.0 a	5.0 ± 0.3 cd	1.0 ± 0.1 e
20.0	0.0	70.9 ± 10.5 cd	6.6 ± 0.7 ab	1.8 ± 0.1 bcd
	20.0	83.3 ± 7.4 bc	3.2 ± 0.3 e	2.7 ± 0.2 a
Significance		**	**	**

²Mean values (n = 30) (±SE) in each column followed by the same lowercase letter do not differ significantly at $P \leq 0.05$ according to Student's *t* test.

**Significant at $P \leq 0.01$.

BA = benzyladenine; IBA = indole-3-butyric acid.

Table 5. Effects of NaCl on rooting of *L. monopetalum* microshoots on half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA and NaCl at concentrations shown. Microshoots were excised from the fourth subculture on a medium without NaCl.

NaCl (g·L ⁻¹)	Callusing (%)	Rooting (%)	Mean root number	Mean root length (cm)	Survival (%)
0.0	20.0 ± 6.5 b ²	76.7 ± 2.7 ab	5.8 ± 0.5 a	4.2 ± 0.3 ab	86.7 ± 4.2 ab
1.25	36.6 ± 8.9 a	76.7 ± 6.7 ab	4.0 ± 0.5 b	5.0 ± 0.3 a	86.7 ± 4.2 ab
2.5	0.0 ± 0.0 c	76.7 ± 2.7 ab	4.2 ± 0.4 b	4.0 ± 0.2 b	83.3 ± 3.5 bc
5.0	0.0 ± 0.0 c	86.7 ± 4.2 a	4.0 ± 0.4 b	4.9 ± 0.4 a	93.3 ± 4.4 a
10.0	0.0 ± 0.0 c	80.0 ± 6.5 ab	3.1 ± 0.2 bc	2.6 ± 0.2 c	86.7 ± 5.4 ab
15.0	0.0 ± 0.0 c	63.3 ± 8.2 bc	2.5 ± 0.4 c	2.8 ± 0.4 c	66.7 ± 7.0 c
20.0	0.0 ± 0.0 c	46.7 ± 7.4 c	2.1 ± 0.4 c	2.4 ± 0.4 c	66.7 ± 5.0 c
Significance	**	**	**	**	**

²Mean values (n = 30) (±SE) in each column followed by the same lowercase letter do not differ significantly at $P \leq 0.05$ according to Student's *t* test.

**Significant at $P \leq 0.01$.

IBA = indole-3-butyric acid.

Table 6. Effects of substrate on ex vitro acclimatization of *L. monopetalum* micropropagated plantlets 8 weeks after transplanting.

Substrate (v/v)	Acclimatization (%)	Shoot number per plant	Shoot length (cm)	Root number per plant	Root length (cm)
Peat:perlite 0:1	100.0 ± 0.0 a ²	2.0 ± 0.1 a	2.6 ± 0.1 b	4.6 ± 0.3 b	9.6 ± 0.5 b
Peat:perlite 1:1	100.0 ± 0.0 a	1.9 ± 0.1 a	3.4 ± 0.2 a	5.8 ± 0.3 a	9.8 ± 0.6 ab
Peat:perlite 2:1	100.0 ± 0.0 a	1.6 ± 0.1 a	3.5 ± 0.2 a	5.2 ± 0.4 ab	11.2 ± 0.5 a
Significance	NS	NS	**	*	*

²Mean values (n = 32) (±SE) in each column followed by the same lowercase letter do not differ significantly at $P \leq 0.05$ according to Student's *t* test.

NS, *, **Nonsignificant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

absence of IBA, the application of auxin strongly increased the number of roots (De Klerk et al., 1999). Aiming for better quality of rooted microshoots (i.e., sufficiently long roots and moderate callus formation), IBA at 1.0 mg·L⁻¹ could be preferred for *L. monopetalum*.

Regarding the in vitro response of *L. monopetalum* when subjected to NaCl, during in vitro establishment, the addition of 0.0 to 20.0 g·L⁻¹ NaCl to the medium did not affect shoot formation or number, whereas both shoot length and multiplication index

were gradually decreased as the concentration of NaCl was increased. During subcultures on the same media, the best results regarding the highest shoot number and multiplication index as well as an acceptable shoot length were obtained on medium with relatively low NaCl concentrations (2.5 or 5.0 g·L⁻¹). Higher proliferation rates in the presence of 2.5 to 30.0 g·L⁻¹ NaCl (depending on the species) compared with the control have been reported for the halophytes *Salicornia europaea* (Shi et al., 2006) and *S. brachiata* (Joshi et al., 2012), in contrast to

Crithmum maritimum, whose shoot proliferation was gradually reduced as the concentration of NaCl was increased from 0.3 to 17.6 g·L⁻¹ (Grigoriadou and Maloupa, 2008). Moreover, the growth and development of *Glaux maritima* explants were stimulated by 5.9 g·L⁻¹ NaCl, and *Dianthus arenarius* spp. *arenarius* explants were relatively tolerant to 2.9 g·L⁻¹ NaCl, whereas the growth of *Linaria loeselii* explants was inhibited by 2.9 g·L⁻¹ NaCl (Freipica and Ievinsh, 2010). Shoot length was favored by the addition of NaCl, especially as its concentration was

increased, in accordance with the results that have been reported for *Crithmum maritimum* at 3 to 12 g·L⁻¹ NaCl (Grigoriadou and Maloupa, 2008). During all our experiments with NaCl, shoots did not present any chlorosis or drying, even at the highest NaCl concentration, indicating that *L. monopetalum* is quite tolerant to NaCl.

For in vitro establishment, a medium supplemented with 0.5 mg·L⁻¹ BA and a low concentration (5.0 g·L⁻¹) of NaCl, or without NaCl, could be proposed to achieve the highest shoot number and multiplication index and satisfactory shoot length. In subcultures, a medium supplemented with 0.5 mg·L⁻¹ BA and relatively low NaCl concentration (2.5 or 5.0 g·L⁻¹) provided the best results.

During in vitro rooting, the tolerance of microshoots at NaCl concentration up to 10.0 g·L⁻¹ was revealed because high rooting percentages, comparable to those of the control, were recorded; however, the root number was significantly reduced in all NaCl treatments. At higher NaCl concentrations, not only the rooting percentage but also the survival percentage of microshoots were significantly decreased. Nevertheless, rooting was not completely inhibited, even at the highest concentration of NaCl, as opposed to *Crithmum maritimum* (Grigoriadou and Maloupa, 2008), *Dianthus arenarius* spp. *arenarius*, and *Linaria loeselii* (Freipica and Ievinsh, 2010), in which rooting was significantly inhibited by increasing concentrations of NaCl to 2.9 to 5.9 g·L⁻¹ (depending on the species). In contrast, root development of *Glax maritima* was stimulated by 5.9 g·L⁻¹ NaCl; however, no roots were formed at 23.4 g·L⁻¹ NaCl (Freipica and Ievinsh, 2010).

The effects of NaCl on in vitro propagation of *L. monopetalum* differed for shoot multiplication and rooting phases during which different plant growth regulators were used. During shoot multiplication, with a cytokinin (BA at 0.5 mg·L⁻¹), the addition of a low NaCl concentration was favorable to shoot production; however, during rooting, when auxin (IBA at 1.0 mg·L⁻¹) was used, the addition of NaCl reduced the number of roots produced, and microshoot rooting was satisfactory up to 10.0 g·L⁻¹ NaCl. Shoot tip explants used during the multiplication stage and microshoots cultured for rooting differed only in their length; the shortest shoots were promoted for multiplication and the longest were promoted for rooting. Therefore, it is probable that blastogenesis and rhizogenesis have different sensitivities to NaCl levels. In mangrove *Bruguiera sexangula*, cell proliferation and cell differentiation had different sensitivities to NaCl; 23.4 g·L⁻¹ stimulated the formation of adventitious tissues on leaf calluses but callus growth was inhibited (Mimura et al., 1997). As in our experiments, similar explants were used for shoot multiplication and rooting, and differences in NaCl tolerance at these two micropropagation stages could not be attributed to the differences in explant tissues, which may cause various levels of physiological integration

(Freipica and Ievinsh, 2010). Salt tolerance of the whole plant may differ from that of the tissue culture (Freipica and Ievinsh, 2010), which may be due to the complexity of whole-plant salt tolerance mechanisms (McCoy, 1987).

During acclimatization ex vitro to various peat and perlite mixtures, all transplanted plantlets were successfully acclimatized. Correspondingly, high acclimatization percentages have been reported for other species of the Plumbaginaceae family (Amo-Marco and Ibañez, 1998; Bhadra et al., 2009; Chinnamadasamy et al., 2010; Lubaina et al., 2011; Rout et al., 1999; Sahoo and Debata, 1998; Selvakumar et al., 2001).

In conclusion, the optimum type and concentration of cytokinin for high multiplication rates and satisfactory lengths and quality of *L. monopetalum* shoots were determined. Furthermore, a method of producing multiple rooted microshoots during one stage was proposed. Rooting of microshoots and plantlet acclimatization were highly successful and the rooting capacity of microshoots improved as subcultures progressed. Regarding the effects of NaCl, shoot multiplication was further improved by the addition of relatively low NaCl concentrations (2.5 or 5.0 g·L⁻¹) to the multiplication medium, without a significant increase in shoot elongation. Considering that the explant response for shoot production was satisfactory in media, even without NaCl, it can be suggested that there was no necessity for NaCl as the medium component during this stage; however, it had a synergistic effect on shoot formation. During in vitro rooting, tolerance to NaCl concentrations up to 10.0 g·L⁻¹ was observed because microshoot rooting was not inhibited by the addition of NaCl; however, the root number was reduced as the NaCl concentration was increased.

For in vitro establishment, MS medium supplemented with 0.5 mg·L⁻¹ BA and 0.0 or 5.0 g·L⁻¹ NaCl could be proposed. The best results for shoot multiplication were obtained using a medium with low (0.5 mg·L⁻¹) BA or relatively high (2.0 mg·L⁻¹) kinetin concentrations, which could be further improved by the addition of a low NaCl concentration (2.5 or 5.0 g·L⁻¹) on the 0.5 mg·L⁻¹ BA medium. If the production of multiple rooted microshoots during one stage is desirable, then a medium containing 0.5 mg·L⁻¹ kinetin would be preferred; as a second choice, a medium with 2.0 or 4.0 mg·L⁻¹ 2iP could be used. For in vitro rooting, half-strength MS medium supplemented with 1.0 mg·L⁻¹ IBA could be proposed. For ex vitro acclimatization, a mixture of peat:perlite of 1:1 or 2:1 (v/v) could be used.

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