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Factors influencing axillary bud induction on nodal segments of *Micromeria pulegium* (Rochel) Benth.

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Abstract:

Stojičić, D., Tošić, S., Pavlović, J., Golubović, A., Simonović, J., Zlatković, B.: *Factors influencing axillary bud induction on nodal segments of Micromeria pulegium* (Rochel) Benth. *Biologica Nyssana*, 8 (1), September 2017: 93-98.

Micromeria pulegium (Rochel) Benth. is an endemic species from family Lamiaceae. Plants from this family are characterized by presence of secondary metabolites and antioxidant components. *Micromeria pulegium* contains pulegone which is a potential bio-insecticide and a bio-pesticide. Natural populations of this species are so small that there is a need for an alternative way of propagate and proliferation of individuals. Method of micropropagation was used with the goal of mass production of plants with the chemical composition of essential oils as similar as possible to that in wild-harvested plants. This paper presents the study on influence of concentration of mineral salts, carbon sources (sucrose and maltose) and nitrogen source (casein hydrolysate) on process of *in vitro* regeneration of plants through induction of axillary buds on the nodal segments of *Micromeria pulegium*. The greatest number of axillary buds was formed in explants grown on MS culture medium with 3% sucrose and 500 mg/L casein hydrolysate.

Key words: axillary bud induction, shoot culture, biomass production

Apstrakt:

Stojičić, D., Tošić, S., Pavlović, J., Golubović, A., Simonović, J., Zlatković, B.: *Faktori koji utiču na indukciju aksilarnih pupoljaka na nodalnim segmentima vrste Micromeria pulegium* (Rochel) Benth.. *Biologica Nyssana*, 8 (1), Septembar 2017: 93-98.

Micromeria pulegium (Rochel) Benth. je endemična vrsta iz familije Lamiaceae. Ovu familiju karakteriše prisustvo sekundarnih metabolita i antioksidativnih komponenti. *Micromeria pulegium* sadrži pulegon koji je potencijalni bio-insekticid i biopesticid. Prirodne populacije ove vrste imaju mali broj jedinki pa se nameće potreba za pronalaženjem načina njihovog gajenja i umnožavanja. U cilju masovne produkcije biljaka čiji će hemijski sastav etarskih ulja biti najsljedniji onome koji sadrže biljke iz prirode primenjena je metoda mikropropagacije. U ovom radu ispitan je uticaj jačine mineralnih soli, izvora ugljenika (saharoze i maltoze) i izvora azota (kazein hidrolizata) na proces regeneracije biljaka *in vitro* putem indukcije aksilarnih pupoljaka na nodalnim segmentima *Micromeria pulegium*. Najveći broj aksilarnih pupoljaka je formiran na eksplantatima gajenim na MS hranljivoj podlozi sa 3% saharoze i 500 mg/L kazein hidrolizata.

Ključne reči: indukcija aksilarnih pupoljaka, kultura izdanaka, produkcija biomase

Introduction

Micromeria pulegium (syn. *Clinopodium pulegium*) belongs to section *Pseudomelissa* within the genus *Micromeria* Benth. (family Lamiaceae). This genus includes four sections: *Pseudomelissa*, *Micromeria*, *Cymularia* and *Pineolentia* (Harley et al., 2004). According to molecular evidence, emphasizing similarity of selected morphological traits, Bräuchler et al. (2006) have included species of genus *Micromeria*, sect. *Pseudomelissa*, into *Clinopodium* L. *M. pulegium* is an endemic species of southern Carpathians. Its range includes Romania, Serbia and Federation of Bosnia and Herzegovina. In Serbia it was recorded in the east, in the area of Svrljiški Timok gorge, while previously it used to be present at mountain Tara (Šilić, 1979). Gorge of Svrljiški Timok is situated at low altitude, so summers are very warm and dry, while winters are characterized by strong winds and snow. Habitats are rocky and steep, mostly gorges at 1000-1200 m above sea level. *Micromeria pulegium* is a perennial, medium-sized, erect plant (Fig. 1), and its leaves are densely covered in small glands, rendering a pleasant aroma to the plant. Species of genus *Micromeria* are characterized by presence of secondary metabolites, serving as the foundation of their diverse biological activity (Vladimir-Knežević et al., 2000). These species have antimicrobial properties (Sarac & Ugur, 2007), mostly based on phenol compounds, flavones and flavonoids, terpenoids and alkaloids (Cowan et al., 1999; Cosentino et al., 1999). These aromatic plants have been traditionally used as spices and in alternative medicine (Al-Hamwi et al., 2011; Dudai et al., 2001; Telci et al., 2007). Antioxidant and antimicrobial activities were recorded in both micropropagated plants of *M. pulegium* and those collected from natural habitats (Tošić et al., 2015). Chemical composition of *M. pulegium* essential oils from native and micropropagated plants was also studied (Stojičić et al., 2016). Endemic species with small populations such as those of *M. pulegium*, situated in vicinity of urban environment, are often under negative anthropogenic influence. In order to preserve biodiversity, various biotechnological methods have been developed, and among them micropropagation of plants *in vitro* is highly important (Paunescu, 2009; Reed et al., 2011; Sharma & Sharma,



Fig. 1. Wild-growing plant *M. pulegium*, Svrljiški Timok George

2013). Through micropropagation it is possible to multiply selected genotypes and chemotypes of different plants, avoiding collection from their natural habitat. *In vitro* propagation from field-grown plants through multiplication of nodal segments (axillary shoot formation) is a good method of producing a large number of plants without changing the chemical composition (Santos-Gomes & Fernandes-Ferreira 2003; Affonso et al. 2007). This study was initiated in order to improve the reliable protocol for rapid propagation of *M. pulegium* through axillary bud induction from nodal explants.

Material and methods

Plant material and source of explants

Aerial parts of *M. pulegium* plants, at the vegetative stage of development, were collected from natural populations in Svrljiški Timok gorge, in August 2012 (Fig. 1). Voucher specimen (N° 6912) was deposited in the Herbarium collection of the Faculty of Science and Mathematics, University of Niš (HMN). Nodal segments (one-node stem segments, 1 cm long and bearing two axillary buds) were surface-sterilized for 30 min with 25% solution of sodium hypochlorite (6% active chlorine) containing two drops of liquid detergent. After three rinsing in sterile distilled water, the explants were treated with 5% solution of nystatin for 24 hours in order to eliminate possible fungal infections. After that nodal segments were rinsed three times with sterile distilled water, and they were placed in different variations of culture medium. Every jar closed with polycarbonate cover.

Table 1. Effect of medium strength on *Micromeria pulegium* shoot proliferation, number of shoots per explant, shoot length and shoot fresh and dry weight after 28 days of culture

MS	Explants producing shoots (%)	Number of shoots per explant	Shoot length (mm)	Explant fresh weight (g)	Explant dry weight (g)
0MS	0	0	0	0	0
0.25MS	80.0 ± 0.1 ^a	8.15 ± 0.32 ^a	6.25 ± 0.40 ^a	0.09 ± 0.01 ^a	0.009 ± 0.001 ^a
0.5MS	85.0 ± 0.1 ^b	8.52 ± 0.51 ^a	8.48 ± 0.46 ^b	0.11 ± 0.01 ^b	0.010 ± 0.001 ^a
1MS	93.3 ± 0.2^c	12.32 ± 0.71^b	9.31 ± 0.80 ^c	0.21 ± 0.02^c	0.017 ± 0.002^b
2MS	81.7 ± 0.2 ^a	12.12 ± 0.81^b	9.88 ± 1.11^d	0.22 ± 0.05^c	0.019 ± 0.003^b

Values are mean ± SE, n = 60. Means in the column followed by different letters are different according to LSD multiple range test ($P \leq 0.05$)

Table 2. Effect of carbon source on *Micromeria pulegium* shoot proliferation, number of shoots per explant, shoot length and shoot fresh and dry weight after 28 days of culture

Carbon source (%)	Explants producing shoots (%)	Number of shoots per explant	Shoot length (mm)	Explant fresh weight (g)	Explant dry weight (g)
0	51.7 ± 0.1 ^a	2.33 ± 0.93 ^a	4.93 ± 0.33 ^a	0.06 ± 0.02 ^a	0.002 ± 0.001 ^a
Maltose					
0.5	70.0 ± 0.1 ^b	4.84 ± 0.46 ^b	6.41 ± 0.35 ^{ab}	0.10 ± 0.02 ^{ab}	0.005 ± 0.001 ^b
1.0	75.0 ± 0.1 ^b	4.94 ± 0.66 ^b	6.43 ± 0.48 ^{ab}	0.15 ± 0.03 ^{bc}	0.005 ± 0.001 ^b
3.0	76.7 ± 0.2 ^{bc}	4.60 ± 0.66 ^b	6.05 ± 0.48 ^{ab}	0.11 ± 0.03 ^{ab}	0.006 ± 0.001 ^b
5.0	68.3 ± 0.2 ^b	6.09 ± 0.21 ^c	6.07 ± 0.44 ^{ab}	0.24 ± 0.01^e	0.020 ± 0.001^d
Sucrose					
0.5	80.0 ± 0.1 ^c	9.09 ± 0.21 ^d	7.43 ± 0.44 ^c	0.21 ± 0.01 ^d	0.017 ± 0.001 ^c
1.0	85.0 ± 0.1 ^c	12.52 ± 0.21^e	7.00 ± 0.44 ^c	0.18 ± 0.01 ^c	0.016 ± 0.001 ^c
3.0	93.3 ± 0.2^d	12.32 ± 0.71^e	9.31 ± 0.80^d	0.21 ± 0.02 ^d	0.017 ± 0.002 ^c
5.0	81.7 ± 0.2 ^c	10.09 ± 0.21 ^{de}	6.33 ± 0.44 ^{ab}	0.21 ± 0.01 ^d	0.019 ± 0.001 ^c

Values are mean ± SE, n = 60. Means in the column followed by different letters are different according to LSD multiple range test ($P \leq 0.05$)

Culture medium and culture conditions

Isolated nodal segments were placed horizontally on basal Murashige and Skoog (MS) medium (1962) supplemented with 3% sucrose (w/v) and 0.7% (w/v) agar (Torlak, Belgrade) in 250-mL glass jars containing 25 mL of the medium, if not stated otherwise. Ten explants were placed in each jar. The pH of the media was adjusted to 5.8 prior to autoclaving at 114 °C for 25 min. Cultures were maintained at 25 ± 2 °C under conditions of a 16 h/8 h photoperiod, with a photon flux density 45 μE m⁻² s⁻¹ provided by cool white fluorescent lamps, at 25 ± 2 °C.

Variations of media

Medium strength – the effects of five different medium strengths were tested: 0; 0.25; 0.5; 1.0; or 2.0 times those of MS. The other components in all five media were the same as in MS medium.

Effects of carbohydrates - to determine the influence of different carbon sources, MS medium was supplemented with one of two carbohydrates (sucrose or maltose), each at five different concentrations (0; 0.5; 1; 3; or 5%).

Effect of enzymatic casein hydrolyzate (CH) - as a source of organic nitrogen, CH was tested at the following concentrations 0; 125; 250; 375; or 500 mg/L.

Measured parameters

After 4 weeks in culture, the explants formed axillary buds. The explants reacting positively to treatment were recorded and following parameters were measured. The number of explants producing shoots and number of shoots per explant, as well as explant fresh and dry weight were recorded in order to evaluate the effect of nutritive factors on shoot multiplication. The dry weight of shoots was recorded after drying in separate paper containers for 24 h. Buds shorter than 1 mm were disregarded. The

Table 3. Effect of casein hydrolyzate on *Micromeria pulegium* shoot proliferation, number of shoots per explant, shoot length and shoot fresh and dry weight after 28 days of culture

CH (mg/L)	Explants producing shoots (%)	Number of shoots per explant	Shoot length (mm)	Explant fresh weight (g)	Explant dry weight (g)
0	93.3 ± 0.2^b	12.32 ± 0.71 ^a	9.31 ± 0.80 ^a	0.21 ± 0.02 ^b	0.017 ± 0.002 ^a
125	80.0 ± 0.1 ^a	12.11 ± 0.45 ^a	9.90 ± 0.70 ^a	0.19 ± 0.02 ^a	0.018 ± 0.002 ^a
250	90.0 ± 0.1^b	12.27 ± 0.24 ^a	9.51 ± 0.51 ^a	0.19 ± 0.02 ^a	0.017 ± 0.002 ^a
500	93.3 ± 0.2^b	16.93 ± 0.38^c	15.36 ± 0.57^b	0.25 ± 0.04^c	0.023 ± 0.002^b
750	81.7 ± 0.2 ^a	15.06 ± 0.42 ^b	15.01 ± 1.10^b	0.25 ± 0.02^c	0.024 ± 0.002^b

Values are mean ± SE, n = 60. Means in the column followed by different letters are different according to LSD multiple range test ($P \leq 0.05$)

efficiency of different treatments on growth rate was determined by comparing biomass increase and in vitro proliferation rates, using different methods. Biomass increase was calculated on both fresh and dry weight basis. Proliferation rate was assessed by counting the number of shoots at subculture and following 4-week treatment under previously defined conditions.

Statistical methods

For each treatment, a total of 60 nodal segments (ten explants per jar) were used, divided into two replicates. Data collected from experiments were calculated and statistically analyzed, and differences were tested for significance using ANOVA Multiple range test at the significance level of $P \leq 0.05$.

Results and discussion

Micropropagation is a good method for achieving uniform plant material, and at the same time use of nodal segments for plant regeneration with the goal of mass production is considered a reliable method for many Lamiaceae species (D o d e et al., 2003). In most species of Lamiaceae family, shoot proliferation demands presence of cytokinin in the nutrient medium, with or without auxin (S a h a et al., 2012; B a k h t i a r et al., 2014). However, the nodal explants of *M. pulegium* cultured on MS medium without plant growth regulators have produced shoots (Stojičić et al., 2016). These results were used as a foundation for determining the influence of nutritive factors of nutrient substrate on production of axillary buds on nodal segments of *M. pulegium*. Research on the effects of five different medium strengths has shown that medium without salts and vitamins was not sufficient for development of axillary buds, and necrosis of whole explants happened already in the second week (Tab. 1). On medium supplemented with salts and vitamins, the percentage of explants developing shoots was greater

when concentration of salts increased. The maximum axillary bud proliferation was obtained at 1MS and 2MS, in contrast to results by F a d e l et al. (2010) with *Mentha spicata* L. and Mišić et al. (2006) with *Salvia brachyodon*. They achieved the best results by using medium with MS salts reduced to one half (0.5MS). Supplementation of the medium with salts and vitamins promoted elongation of the shoot. The greatest average length of axillary buds was developed on explants grown at medium with 2MS. The explants with the greatest average fresh and dry weight were grown on 1MS and 2MS medium.



Fig. 2. *In vitro* plantlets cultured on medium with 750 mg/L caseine hydrolysate (left), flowering *in vitro* plantlets on same medium (right)

Explants of *M. pulegium* grown on culture medium MS without a source of carbon were light green, with sporadically developed axillary buds in leaf axils. The percentage of explants with developing axillary buds was the smallest in the whole study (52%). The values of number of buds, length of buds, fresh and dry mass were also significantly smaller than in axillary buds grown in medium supplemented with sugar (Tab. 2). Sucrose is often the best source of carbon in micropropagation of various species from family Lamiaceae (S u j a n a & N a i d u, 2011). After adding maltose or sucrose

sugars to the nutrient medium, the percentage of *M. pulegium* explants with axillary buds has significantly increased (68-93%). The explants grown on medium with sucrose were healthy, with good branching, green, with elongated internodes and a large number of developed axillary buds. The results of our study show that sucrose was a better source of carbon for *M. pulegium* than maltose. In all studied concentrations sucrose caused a greater number of axillary buds than when the medium contained maltose. The maximum number of buds developed on medium with 1% and 3% sucrose, while the greatest length was recorded in axillary buds growing on medium with 3% sucrose. The explants grown on medium with maltose had bush-like form with shorter internodes. Therefore the greatest values of average dry and fresh biomass were recorded in explants grown on medium with the greatest concentration of maltose.

Casein hydrolysate is commonly used in micropropagation as a source of nitrogen. Most of the recorded effect was stimulative (Stojičić et al., 2008). Explants of *M. pulegium* grown on MS medium with or without casein hydrolysate showed no morphological differences. They were elongated, branching explants, dark green in color, with a large number of developed axillary buds (**Fig. 2**). The stimulative effect of casein hydrolysate was manifested in increase of length of axillary buds and their fresh and dry biomass (**Tab. 3**). On medium with the lowest concentrations of casein hydrolysate this increase was not statistically significant. However, use of casein hydrolysate in concentrations of 500 and 750 mg/L is justified as it leads to statistically significant increase in bud length and biomass. The maximum length of axillary buds was recorded in explants grown on medium with 500 and 750 mg/L of casein hydrolysate. Some of the explants grown on this medium produced flowers (**Fig. 2**). In some of the explants grown on medium with the greatest concentration of casein hydrolysate adventive roots have formed spontaneously without addition of auxin to the medium. Root formation without auxin was observed in a number of Lamiaceae species (Zuzarte et al., 2010; Bassolino et al., 2015), where roots were induced on plant growth regulator free medium. However, in *M. pulegium* shoots rooted spontaneously on the plant growth regulator free medium; hence, the auxin treatment was used to promote rooting (Stojičić et al., 2016).

Conclusion

Micromeria pulegium (Rochel) Benth. is characterized by relatively high amounts of the

essential oils rich in pulegone that potentially may be used as bio-insecticide and bio-pesticide (Koul et al. 2008). Specimens collected in the wild and grown *in vitro* both produced essential oil of relatively stable composition (Stojičić et al., 2016). Careful selection of the culture conditions may increase accumulation of biomass and production of secondary metabolites, which may be employed to obtain essential oils for commercial use. Results indicate that micropropagation of *M. pulegium* is influenced by composition of nutritive medium. Selection of proper mineral salt concentration, carbon source and especially presence of nitrogen source were shown to be important for stimulation of development and growth of axillary buds in this species. The presented protocol may be used as a foundation in further research in order to produce *M. pulegium* plants with desired characteristics, especially for production of secondary metabolites.

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