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Micropropagation of *Micromeria juliana* (L.) Benth. ex Rchb. (Lamiaceae)

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***Abstract*:**

Tošić, S., Nikolić, S., Jovanović, M., Zlatković, B., Stojičić, D.: *Micropropagation of Micromeria juliana (L.) Benth. ex Rchb. (Lamiaceae). Biologica Nyssana, 6 (1), September 2015: 17-23.*

Micromeria juliana belongs to family Lamiaceae, whose representatives are characterized by a significant level of essential oils and antioxidant components. Several species of genus *Micromeria* contain biologically active substances and are therefore used in folk medicine, food industry and cosmetic industry. Methods of their tissue culture may provide rapid mass multiplication of plants for various purposes, including research on production, accumulation and metabolism of important secondary metabolites. The goal of this paper was to determine the protocol for regeneration of *M. juliana* plants through use of nodal explants on nutritive substrate with various growth regulators. The greatest number of axillary buds was formed in explants grown on MS nutritive medium with 3 μM benzyladenine (BA) and 0.57 μM indole-3-acetic acid (IAA). The explants grown at the medium without any growth regulators and the medium with auxin have shown spontaneous root formation.

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

***Apstrakt*:**

Tošić, S., Nikolić, S., Jovanović, M., Zlatković, B., Stojičić, D.: *Micropropagacija vrste Micromeria juliana (L.) Benth. ex Rchb. (Lamiaceae). Biologica Nyssana, 6 (1), Septembar 2015: 17-23.*

Micromeria juliana (L.) Benth pripada familiji Lamiaceae koja se odlikuje značajnim sadržajem etarskih ulja i antioksidativnih komponenti. Vrste roda *Micromeria* sadrže biološke aktivne supstance zbog čega se koriste u narodnoj medicini, kao i u prehrambenoj i kozmetičkoj industriji. Metode kulture tkiva mogu da obezbede brzu i masovnu multiplikaciju biljaka za različite svrhe kao što je proučavanje produkcije, akumulacije i metabolizma značajnih sekundarnih metabolita. Cilj ovog rada je da ustanovi protokol za regeneraciju biljaka *M. juliana* korišćenjem nodalnih eksplantata, na hranljivoj podlozi sa različitim regulatorima rastenja. Najveći broj aksilarnih pupoljaka formiran je na eksplantatima gajenim na MS hranljivoj podlozi sa 3 μM benziladenina (BA) i 0,57 μM indol-3-sirćetne kiseline (IAA). Eksplantati gajeni na podlozi bez regulatora rastenja i na podlozi sa auksinom su spontano ožiljavali.

Key words: indukcija aksilarnih pupoljaka, kultura izdanaka, produkcija biomase

Introduction

In relation to the morphological characteristics and phylogenetic relationships, species of genus *Micromeria* are grouped into three sections: *Cymularia*, *Micromeria* and *Pseudomelissa*. The representatives of this genus in the flora of Serbia (Diklić, 1974) belong to *Micromeria* (*M. croatica*, ***M. juliana***, *M. cristata* and *M. parviflora*) and *Pseudomelissa* (*M. thymifolia*, *M. albanica* and *M. pulegium*). *M. juliana* is comparatively rare species in Serbia, recorded only in the vicinity of Kačanik in Kosovo and Metohija province (Diklić, 1974). According to Chater & Guinea (1973) and Šilić (1979) the native range of the species includes countries of Mediterranean region (Montenegro, Croatia, Herzegovina, Macedonia, Greece, Bulgaria, Albania, France, Portugal, Italy, Turkey, northwestern Africa, as well as the western part of Asia Minor and Crete). It inhabits dry, sunny sand-based habitats, stony ground and crevices in limestone rocks (**Fig. 1**). This is a perennial, dwarf shrubby plant, with many erect, mostly simple stems, up to 40 cm in height. The leaves are narrowly ovate, upper linear-lanceolate, entire, with revolute margins and opposite. The root and the basal part of stems are somewhat fruticose. Flowers are comparatively small, purple in sessile or shortly pedunculate verticillasters, organized into long, common inflorescences (Doroszenko, 1986).

The representatives of genus *Micromeria* are characterized by low morphological diversity and their growth and development are influenced by numerous ecological factors, leading to high diversity of their secondary metabolites. Due to presence of secondary metabolites, species of genus *Micromeria* show antioxidant (Güllüce et al., 2004; Stojanović & Palić, 2008; Vladimir-Knežević et al., 2011;), antibacterial (Tabanca et al., 2001; Duru et al., 2004; Sarac & Ugur, 2007; Stojanović & Palić, 2008), antifungal (Marinković et al., 2003; Abou-Jawdah et al., 2004; Özcan, 1999), antiphytoviral (Bezić et al., 2013), insecticidal (Aslan et al., 2005), bioherbicidal (Dudai et al., 1999) and allelopathic (Dudai et al., 2009) biological activity. Due to their pharmacological properties, plant species of genus *Micromeria* are used in folk medicine for treating gastrointestinal and respiratory tract, skin infections and wounds, as well as for soothing pain and preventing insomnia (Šarić-Kundalić et al., 2011). They are also used in cooking in order to improve taste and aroma of food, as additives and spices (Tabanca et al., 2001; Güllüce et al., 2004).

Fig. 1. *Micromeria juliana*, canyon of river Morača (Montenegro)

The high individual variability characterizing species of genus *Micromeria* is a limiting factor for the intensity of their use in pharmaceutical purposes. The solution to this problem may include use of plant tissue cultures *in vitro* (Saha et al., 2012). The methods of culture *in vitro* are important in reproduction of endemic and rare plants, contributing to preservation of biodiversity (Fay 1992; Reed et al., 2011). Growing in culture *in vitro* decreases the exploitation pressure on natural populations and in a short period of time may yield several thousand plants from a small amount of plant samples. The only species of genus *Micromeria* previously grown in culture *in vitro* used to be *M. pulegium* (Tošić et al., 2015).

Material and methods

Plant material and source of explants

The surface sterilization included treating seeds with 30% solution of Na-hypochlorite with 4% of active chlorine for 25 minutes. After three rinsing in sterile distilled water, the seeds were treated with 5% solution of nystatin for 24 hours in order to eliminate possible fungal infections. Next step included rinsing seeds in sterile distilled water three times, and then they were placed individually in test tubes on nutritive medium in aseptic conditions in order to germinate. After the seeds have germinated, the resulting plants were used for preparing explants – nodal segments. In order to introduce *M. juliana* into *in vitro* culture, seeds were collected from plants in their natural habitat in canyon of river Morača (Montenegro). A voucher specimens, under the acquisition number 10850, are deposited at the herbarium collection of Department of Biology and Ecology, Faculty of Science and Mathematics in Niš (HMN).

Culture medium and culture condition

The nutritive medium used in culture *in vitro* was Murashige & Skoog (1962) – MS medium, including appropriate macro- and micro-mineral salts and organic additions. All medium have included 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 7 g l^{-1} agar. The pH value of each medium was checked immediately before autoclaving and if necessary adjusted to pH 5.8 by addition of 0.1 N NaOH. The sterilized nodal segments of *M. juliana* of approximately same size (5 mm) were placed on nutritive medium: without any growth regulators, with cytokinin BA (0.1, 0.3, 1, 3, 10 μM), with auxin IAA (0.57μM), and with combination of BA and IAA. Each medium was used for 30 explants, 10 in each of three jars. The plants were maintained at the temperature 23˚C ± 3˚C and photoperiod of 16 hours of light and 8 hours of dark. The density of light flux was 47 μmol $s^{-1}m^{-2}$.

Measured parameters

The explants were grown for four weeks until they formed axillary buds. The explants reacting positively to treatment were recorded and following parameters were measured: number of axillary buds per explant, length of each bud, fresh and dry mass of each explant. Buds shorter than 1 mm were disregarded. After the fresh mass was measured, explants were dried in separate paper containers and dry mass was then measured by using the same analytic scales. Shoot forming capacity (SFC) index was calculated according to Martinez-Pulido et al. (1992) as follows: SFC index = % explant with shoots x mean number of shoot per explants / 100.

Statistical methods

Data processing was performed by using the statistically-graphic package Statgraphics, procedure ANOVA and test LCD at the significance level $p<0.05$.

Results and discussion

Culture of *M. juliana* was formed by germinating seeds on MS nutritive medium. Use of MS nutritive medium produces favorable results for *in vitro* germinating of numerous species (Molia & Kwapata, 2000), including many species of Lamiaceae. Nodal segments were isolated from *in vitro* germinated seedlings and placed in inductive medium. The initiation of the culture was achieved according to the same procedure, using nodal segments of seedlings, in *Clinopodium odorum* (Diaz et al., 2012). Use of nodal segments for regeneration of plants with the goal of mass production is considered a simple method for many species of Lamiaceae, and at the same time it is the most reliable method for achieving uniform plant material (George & Sherrington, 1984; Dode et al., 2003).

All explants on the MS nutritive medium without regulators (100%) have developed axillary buds (**Tab. 1**). The smallest ratio of explants with axillary buds (63.3%) was recorded at the medium with 0.57 μM IAA.

At the explants grown on medium without growth regulators there were on average 4.97 buds per explant. Most buds were formed on explants grown on medium with 3 μM BA and 0.57 μM IAA. Their number is statistically significantly greater than the number of buds formed on explants on all other medium. Explants from this medium are characterized with the greatest multiplication index of 8.73. After three months, one explant may produce 3665 buds which potentially may produce new plants. Low-concentration auxin combined with cytokinin often assists development of buds on explants (Tejavathi & Indira, 2011).

The greatest average length of axillary buds was developed on explants grown at medium with 0.57 μM IAA (**Tab. 1**). All concentrations of BA used in this study with or without auxin have inhibited elongation of axillary buds in *M. juliana*. The inhibitory effect of high cytokinin concentrations on elongation of buds was recorded in *Mentha piperita* (Ghanti et al., 2004) and *Melissa officinalis* (Tavers et al., 1996). Cytokinins may show inhibitory activity on elongation of axillary buds in spite of promoting their proliferation (van Staden et al., 2008).

The explants with the greatest average mass were grown on MS medium with 3 μM BA and 0.57 μM IAA (**Tab. 2**). On that medium they formed the greatest number of buds, contributing to the greatest biomass. The explants from these medium were healthy, with good branching, green, quite uniform in stature, with shorter internodes and almost bush-like habitus (**Fig. 2**). Combination of cytokinin and auxin was used by numerous authors in order to induce bud formation. For example in *Agastache rugosa* the greatest number and best quality of buds was achieved on MS medium with this particular combination of hormones (Zielinska et al., 2011). The comparison of explants of *M. juliana*

Table 1. Effect of plant growth regulators on *Micromeria juliana* shoot proliferation, number of shoots per explants and shoot length after 28 days of culture.

PGR (µM)		**Explants producing shoots (%)**	**Shoots per explant**	**Shoot length (mm)**	**SFC index**
BA	**IAA**				
-	-	100	4,97 ± 0,50^{abc}	3,95 ± 0,31^{bc}	5,86
0.1	-	96.7	5,86 ± 0,40^{bcd}	3,25 ± 0,27^{ab}	6,97
0.3	-	100	6,00 ± 0,36^{de}	3,18 ± 0,24^{ab}	5,72
1	-	96.7	7,21 ± 0,43^{e}	3,19 ± 0,20^{ab}	3,82
3	-	83.3	5,72 ± 0,38^{cd}	2,72 ± 0,19^{a}	4,14
10	-	70.0	3,95 ± 0,49^{ab}	2,46 ± 0,23^{a}	3,28
-	0.57	63.3	4,68 ± 0,33^{abcd}	**6,47 ± 0,79^{d}**	2,96
0.1	0.57	100	5,73 ± 0,42^{d}	4,12 ± 0,37^{c}	5,73
0.3	0.57	93.3	3,68 ± 0,49^{a}	4,18 ± 0,42^{c}	3,43
1	0.57	96.7	4,52 ± 0,42^{abc}	3,94 ± 0,36^{bc}	4,37
3	0.57	93.3	**9,39 ± 0,65^{f}**	3,84 ± 0,20^{bc}	**8,73**
10	0.57	100	4,37 ± 0,38^{ab}	2,56 ± 0,18^{a}	4,37

The values are means ± standard error. Means within the column of each factor followed by the same letter are not significantly different according to LSD multiple range test ($p \leq 0.05$)

Table 2. Effect of plant growth regulators on *Micromeria juliana* biomass production (using nodal shoot segments as explants) after 28 days of culture.

PGR (µM)		Biomass fresh weight per explant (mg)	Biomass dry weight per explant (mg)
BA	IAA		
-	-	0.017 ± 0.0019^{a}	0.0028 ± 0.00030^{a}
0.1	-	0.022 ± 0.0016^{a}	0.0031 ± 0.00019^{a}
0.3	-	0.021 ± 0.0013^{a}	0.0031 ± 0.00021^{a}
1	-	0.022 ± 0.0016^{a}	0.0034 ± 0.00024^{ab}
3	-	0.015 ± 0.0010^{a}	0.0022 ± 0.00015^{a}
10	-	0.014 ± 0.0014^{a}	0.0020 ± 0.00021^{a}
-	0.57	0.046 ± 0.0079^{cd}	0.0056 ± 0.00066^{bcd}
0.1	0.57	0.027 ± 0.0019^{ab}	0.0043 ± 0.00027^{abc}
0.3	0.57	0.044 ± 0.0053^{c}	0.0057 ± 0.00066^{cd}
1	0.57	0.051 ± 0.0013^{cd}	0.0060 ± 0.00057^{cd}
3	0.57	**0.061 ± 0.0042^{d}**	0.0070 ± 0.00041^{de}
10	0.57	0.038 ± 0.0034^{bc}	**0.0085 ± 0.00074^{e}**

The values are means ± standard error. Means within the column of each factor followed by the same letter are not significantly different according to LSD multiple range test ($p \leq 0.05$).

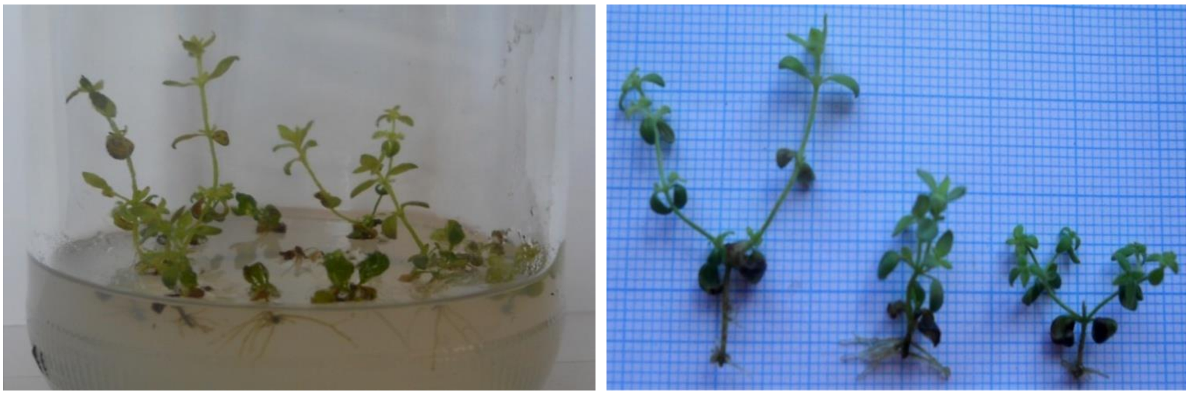

Fig. 2. *M. juliana* on MS medium with 0,57 µM IAA

grown on medium with BA and explants grown on medium with combination of cytokinin and auxin has shown an obvious prominent increase in biomass yield in medium supplemented with auxin. Low-concentration auxin and BA have synergic effect contributing to formation of biomass in explants of *M. juliana*. BA and IAA also had positive effect on bud induction in species *Ocimum killimandscharicum* L. (Sharma et al., 2013), *Ocimum citriodorum* Vis. (Janarthanam & Sumathi, 2012), and in species *Mentha viridis* L. (Rahman et al., 2013). Medium with combination of auxin and cytokinin was effective for regeneration of *Ocimum basilicum* (Nirmal & Sehgal, 1999) and *Salvia brachyodon* (Misic et al., 2006).

Of the explants grown on medium without growth regulators, a small number has spontaneously developed adventive roots, but almost all explants grown on medium with auxin and without cytokinin have developed adventive roots, indicating strong role of auxin in development of adventive roots (**Fig. 2**).

Conclusion

Micromeria juliana (L.) Benth. ex Rchb. was successfully introduced into *in vitro* culture. Results of this study have shown that the maximum number of axillary buds (9.39) was formed in explants grown on nutritive medium with 3 µM BA and 0.57 µM IAA. The nutritive medium with 0.57 µM IAA was the most efficient in elongation of axillary buds, and the average bud length was 6.47 mm. Combination of auxin and high concentrations of BA (3 and 10 µM, respectively) induced formation of the greatest fresh and dry mass, respectively. Root formation in nodal explants took place in medium without growth regulators and in medium that contained both auxin and low concentration of BA. With the increase in concentration of BA there was a decrease of the number of explants developing adventive roots.

This study has shown that the described micropropagation protocol enabled production of a substantial number of plantlets, starting with a small quantity of plant material. In addition, micropropagation of *M. juliana* may be considered as an alternative method for production of secondary metabolites.

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