

Efficient micropropagation and rooting of *Micromeria croatica* (Pers.) Schott (*Lamiaceae*)

Učinkovita mikropropagacija i zakorjenjivanje vrste *Micromeria croatica* (Pers.) Schott (*Lamiaceae*)

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EFFICIENT MICROPROPAGATION AND ROOTING OF *Micromeria croatica* (PERS.) SCHOTT (*Lamiaceae*)

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SUMMARY

Micromeria croatica is an endangered species with a great potential for use as a medical as well as ornamental plant. For commercial use, an efficient propagation protocol is required. The aims of this study were to establish an efficient protocol for micropropagation and rooting of *M. croatica*. A number of factors were tested including the influence of 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) at different concentrations as well as the effect of explant orientation and topophysis on shoot proliferation. Rooting was investigated in agar-based medium supplemented with indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA) at various concentrations. Media with 1 and 2 mg/L of BAP produced the highest number of shoots/explant. However, the lower concentration of BAP should be recommended due to longer shoots without any sign of hyperhydration. Explant orientation and node position showed equal capability for shoot proliferation. Importantly, 100% efficiency of rooting was achieved in an agar-based rooting medium. Acclimatisation exceeded 90%. Taken together, an efficient protocol for micropropagation and rooting of *M. croatica* has been established for future production.

Key-words: *in vitro* propagation, *Micromeria croatica*, plant growth regulators, topophysis

INTRODUCTION

Micromeria croatica (Pers.) Schott (*Lamiaceae*) is a perennial shrub species with long stems (20-30 cm), stalkless leaves, and either pink-purple or white flowers appearing June to August. It is an endemic species to the Dinaric Alps, growing in crevices of calcareous rocks at altitudes of 150-2000 m (Šilić, 1984). Like other species from the *Lamiaceae* family, *M. croatica* is a rich source of essential oils, whose representative constituents are caryophyllene oxide and E-caryophyllene (Kremer et al., 2012). The presence of these essential oils suggests that *M. croatica* could be useful in the treatment or even prevention of human diseases in which free radicals and other reactive oxygen species have been implicated (Vladimir-Knežević et al., 2011). The use of *M. croatica* has been previously tested with ethanolic extract of *M. croatica* found to ameliorate

oxidative stress, inflammation, and fibrogenesis in experimental mice. Furthermore, hepatoprotective and antifibrotic effects of *M. croatica* were comparable to those of silymarin, a well-known standard hepatoprotectant (Vladimir-Knežević et al., 2015). These antioxidant effects have been attributed to high level of phenolic compounds, especially of rosmarinic acid in the *M. croatica* extract. Furthermore, it has also been found out that the leaf extract of *M. croatica* exhibited protective properties against reactive oxygen species-induced DNA damage and inhibited lipid peroxidation (Šamec et al., 2015). Therefore, due to these preliminary but

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promising results of the medicinal properties of *M. croatica* extract, interest in this species will increase and further research is required. Use of the species as an ornamental plant suitable for small rocky gardens should be considered as well (Karlović et al., 2011).

However, *M. croatica* has been an endangered species since 2006 and is accordingly a protected species in Croatia, with any collecting of this species from nature leading to possible extermination. Therefore, in the case of commercial use of this species, an efficient propagation protocol is required. Previously it has been noted that vegetative propagation *in vivo* by cuttings was of low success (D. Kremer, 2014, personal communication). *In vitro* propagation by axillary branching of different species from the *Lamiaceae* family was successful (Mehta et al., 2012; Tošić et al., 2015; Bakhtiar et al., 2016; Vinh et al., 2017). Tošić et al. (2015) propagated *M. croatica* using seedlings obtained *in vitro* as material for culture establishment. However, cloning initiated from adult plant specimen is important when superior chemotaxonomical and/or morphological traits have to be retained for commercial production.

Therefore, the goals of this study were (1) to establish *in vitro* culture of *M. croatica* from adult plant specimen, (2) to determine the effect of the composition of plant growth regulators (PGRs) as well as the effect of explant orientation on axillary shoot proliferation, (3) to determine the effect of topophysis on axillary shoot proliferation, and (4) to investigate the efficiency of rooting micropropagated shoots.

MATERIAL AND METHODS

Culture establishment

As the initial plant material, a single plant provided by the Botanical Garden "Fran Kušan" was used, originally collected from the Velebit nature park (Kuk of Špiljić plane, 44°53' N, 15°16' E, and 1232 m above sea level). The plant was grown in a pot in the greenhouse until multiple stems of the shrub reached about 10 cm. For surface sterilisation, those stems were washed under tap water for 15 min, immersed in 70% ethanol for 1 min, and then immersed again for 15 min in a solution consisting of 1.5% sodium hypochlorite (1.5% active chlorine), 0.1% Tween 20 (v/v), and 150 mg/L of ascorbic acid. After that the stems were rinsed four times with sterile distilled water supplemented with 150 mg/L of ascorbic acid. The surface-sterilised stems were, then, used for explant preparation. Apical buds (about 2 mm long) or one-nodal segments without leaves were cut off and placed on the establishment medium (EM) and cultured in a growth chamber. EM medium consisted of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol, 1 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L 1-naphthaleneacetic acid (NAA), 1 g/L activated charcoal, 150 mg/L ascorbic acid, and 8 g/L agar (Difco Bacto, USA), with the pH adjusted to 5.8. A number of

subcultures were made on EM (without activated charcoal and ascorbic acid from the second subculture) in order to produce enough material to set up experiments.

Shoot proliferation

Influence of PGRs constitution and explant orientation on shoot proliferation. After establishment, one-nodal segments with leaves were placed vertically or horizontally on the proliferation medium (PM), consisting of MS medium, 30 g/L sucrose, 0.1 g/L myo-inositol, and 8 g/L agar, with the pH adjusted to 5.8. The PM was supplemented with different concentrations of BAP (0.5-2.0 mg/L) alone or in combination with NAA (0.2-1.0 mg/L). In addition, PM medium without any hormone (HFMS) was used as a control. Callus formation and the number of microshoots as well as their length were estimated after each subculture lasted for 45 days.

Influence of node position on the shoot proliferation. In additional experiment, one-nodal segments, from the 1st and 5th node (numbered basipetally) of the uniform shoots (approximately 4 cm long) were cut off and placed vertically in PM supplemented with 0.5 mg/L BAP and 0.2 mg/L NAA. Number of microshoots and their length in this experiment were recorded after 28 days.

Rooting

Microshoots approximately 1-2 cm long were taken from PM supplemented with 1 mg/L BAP. Shoots were placed in agar-based media being either HFMS or HFMS supplemented with different concentrations of indole-3-butyric acid (IBA) (0.5-1.5 mg/L) or indole-3-acetic acid (IAA) (0.5-1.5 mg/L). All together seven different treatments of agar-based rooting medium (RM) were applied. The percentage of rooting, number of roots/shoot, length of the longest root (mm), the mean number of shoots per shrub, and the length of the longest shoot were recorded after 45 days. Rooted plantlets were transferred to a sterile substrate (50% peat, 50% river sand) filled in ordinary pots and acclimatised in the growth chamber. Throughout all experiments the conditions in the growth chamber were 22°C with a 16 h photoperiod of cool white light (40 μE/m²/s).

Experimental design and statistical analysis

All the experiments were set up in a completely randomised design. For shoot proliferation, one experimental unit was considered one explant. For the percentage of rooting, the experimental units were Magenta vessels with 9 plantlets. The effect of treatments (PGRs constitution) on shoot proliferation was monitored for three subcultures with 18 explants inoculated per each treatment in each subculture (54 explants per treatment in total). Within each treatment half of the explants were placed vertically and other half horizontally. The topophysis effect was estimated from 90 explants of each node type (1st or 5th). Data from rooting were collected from two successive experiments, each containing 18

plantlets per treatment. The main effects of the variable tested (PGRs constitution and explants orientation) and their role in shoot proliferation were determined by the General Linear Model, two-way analysis of variance (ANOVA). The effects of explant topophysis on shoot proliferation and rooting were analysed by one-way ANOVA. Bonferroni tests at $P < 0.05$ were performed for mean value comparisons. All analyses were performed using SAS version 9.2 (SAS Institute, 2011).

RESULTS AND DISCUSSION

Culture establishment

The apical buds survived culture establishment and were able to form elongated shoots from 94% of inoculated explants and one-nodal segments from 23% of explants. In addition, no contamination was recorded. Ascorbic acid and activated charcoal prevented phenolic compounds oxidation and contributed to the survival of explants. As it is important to maintain superior morphological and/or chemotaxonomical traits in the case of commercial production, the process of cloning was initiated from adult plant specimen. Plantlets established from seed germination *in vitro* could also be source of responding explants as shown by Tošić et al. (2015), however those plants will not be consistent due to the genotypic heterogeneity of the zygotic embryos. In this study, explants survived establishment were subcultured several times on EM medium, until a sufficient number of shoots were obtained to set up the experiment.

Shoot proliferation

Influence of PGRs constitution and explant orientation on the shoot proliferation. There were 13 different treatments tested, 1 hormone free and 12 varying compositions of PGRs in PM were used in the study of their influence on axillary shoot proliferation. It was found out that the number of microshoots and their length significantly ($P < 0.001$) depended upon the different compositions of PGRs in the medium. However, neither explant orientation nor the interaction of these two factors showed any significant influence on the axillary shoot proliferation. All the treatments produced axillary shoots, but the highest number (18.5 shoots) of long enough shoots/explant were obtained on the 1 mg/L BAP medium, making this study most successful to date (Table 1, Figure 1a). This number of shoots is six times higher than it has been previously found for other species of the *Lamiaceae* family such as *Mentha piperita* (Mehta et al., 2012.), *Thymus persicus* (Bakhtiar et al., 2016), and *Lavandula angustifolia* (Vinh et al., 2017). In addition, the previous attempts at micropropagating *M. croatica*, and closely related species, *Micromeria juliana*, obtained 10.34 and 9.39 shoots/explant on MS medium containing 1 mM kinetin and 3 mM benzyladenine plus 0.57 mM IAA, respectively (Tošić, 2015; Tošić et al., 2015). However, it should be noted that counting of new microshoots in this study was performed after 45 days, while previously it has been performed after four weeks. Therefore, it seems that the extra time of subcultivation had an increased effect of axillary shoot proliferation in *M. croatica*.

Table 1. Efficiency of the shoot proliferation of *Micromeria croatica* depending on the constitution of PGRs

Tablica 1. Efikasnost proliferacije izdanaka vrste *Micromeria croatica* u ovisnosti o sastavu biljnih regulatora rasta

PGRs constitution (mg/L) Sastav biljnih regulatora rasta (mg/L)		No. of shoots/explant Broj izdanaka/eksplantatu	Shoot length (mm) Dužina izdanaka (mm)	Callus diameter (cm) Promjer kalusa (cm)
BAP	NAA			
0	0	2.9d	5.0c	0.0
0.5	0	13.7abc	9.2ab	0.0
0.5	0.2	9.4abcd	10.9a	0.5-1.0
0.5	0.5	8.2bcd	6.8bc	0.5-1.0
0.5	1.0	6.7cd	4.0c	0.5-1.0
1.0	0	18.5a	11.3a	< 0.5
1.0	0.2	11.7abcd	7.9abc	< 0.5
1.0	0.5	7.7cd	7.1abc	0.5-1.0
1.0	1.0	6.6cd	4.7c	0.5-1.0
2.0	0	18.2ab	6.9abc	0.0
2.0	0.2	15.8abc	8.0abc	< 0.5
2.0	0.5	13.2abc	6.4bc	0.5-1.0
2.0	1.0	11.2abcd	3.7c	0.5-1.0

Values within the column followed by the same letter are not significantly different according to Bonferroni tests at a $P < 0.05$

Almost identical results were obtained for 2 mg/L BAP. However, the shoots were shorter when grown on this treatment (Table 1). Moreover, the concentration of cytokinins under these growth conditions caused hyperhydration in a certain number of shoots clusters. Though it is known that a higher concentration of PGRs can lead to an increased risk of somaclonal variation, it is most suitable to use a lower concentration of BAP (Bairu et al., 2011; Viehmannova et al., 2016). In addition, callus formation tended to be higher in media where NAA was used in combination with BAP. Increase in concentration of NAA from 0.2-1 mg/L also decreased the number of shoots/explants (Table 1).

On the other hand, explant orientation (vertical or horizontal) did not influence the number of new shoots produced. The average number of shoots/explants across all the treatments was 11 regardless of whether they were set horizontally or vertically. This has been previously seen in a study of axillary shoot induction between node segments of *Cosmos artrosanguineus* placed vertically or horizontally (Kozak et al., 2013). In this study the explants were quite short (one-nodal) and therefore as a consequence the buds all had a similar exposure to the PGRs, regardless of their orientation, which most likely diminished the possible influence that explant orientation may have on proliferation.

Influence of node position to the shoot proliferation. The different node position (1st and 5th) used as an explant was found out to have no statistical significance on the success of micropropagation of *M. croatica*. The average number of shoots/explant after 28 days in culture was 5.1 on the 1st node and 5.2 on the 5th node. Length of the shoots was also similar and amounted 9 mm. The positional effects on subsequent growth or development of explants or topophysis can be quite large, and therefore an understanding of topophysis for optimised selection of initial explants can significantly improve the efficiency of a micropropagation system (George and Sherrington, 1993; Lee-Espinosa et al., 2008).

Previously, it has been found out that *in vitro* cultured axillary rose buds from the medial section of the parent shoot developed more rapidly than either apical or basal buds (Bressan et al., 1982). Furthermore, it has been previously found out that growth from axillary buds of roses increased acropetally (Zieslin et al., 1976). However, the results in this study correlate to earlier work that found out equal capability for shoot proliferation (on medium supplemented with 6-benzyladenine) of younger, 4th or 5th, as well as older cotyledonary nodes in the organogenic culture of eucalypt (*Corymbia torelliana* × *C. citriodora*) (Hung and Trueman, 2011). Though they found out strong, negative morphogenic gradients in shoot elongation, proliferation and rooting from the cotyledonary to the 4th or 5th node of the seedlings their nodes were transferred to the node culture without 6-benzyladenine.

Rooting

It has been previously established that a number of species from the *Lamiaceae* family are very conducive for *in vitro* rooting, with very high rooting efficiency found for *Ocimum basilicum*, *Mentha piperita*, and *Thymus persicus* (Asghari et al., 2012; Mehta et al., 2012; Bakhtiar et al., 2016). In correlation with these, there was 100% efficiency of rooting microshoots of *M. croatica* in both HFMS and RM supplemented with 0.5-1.5 mg/L IBA and 0.5 mg/L IAA (Table 2, Figure 1b). However, lower root induction was seen in explants grown in agar-based medium supplemented with 1 mg/L IAA. Additionally, roots were found out to be the longest when microshoots were rooted in RM supplemented with 1 mg/L IBA. Importantly, microshoots continued to develop axillary branching during the rooting process as seen in Figure 1c. Furthermore, micro-shrubs were calculated to have on the average 3.8 shoots when grown in HFMS medium (Figure 2). This is extremely desirable, as *M. croatica* is a naturally bushy plant and potential use of this species either as ornamental or for medical purposes would require as higher branching as possible.

Table 2. Efficiency of rooting in agar-based rooting medium using varying compositions of PGRs

Tablica 2. Efikasnost zakorjenjivanja u mediju s agarom, uz različit sastav biljnih regulatora rasta

PGRs constitution (mg/L) Sastav biljnih regulatora rasta (mg/L)		Rooting response (%) Uspješnost zakorjenjivanja (%)	No. of roots/shoot Broj korjenčića/izdanku	Length of the longest root (mm) Dužina najdužega korijena (mm)
IBA	IAA			
0	0	100.0a	2.6a	18.6bc
0.5	0	100.0a	2.3ab	17.6bcd
1.0	0	100.0a	2.3ab	24.1a
1.5	0	100.0a	1.3c	22.3ab
0	0.5	100.0a	1.9bc	19.1ab
0	1.0	78.0b	2.6a	13.5cd
0	1.5	93.0a	1.6c	12.9d

Values within the column followed by the same letter are not significantly different according to Bonferroni tests at a $P < 0.05$

Acclimatisation efficiency exceeded 90% when the substrate was sterilised before planting (Figure 1d) and no differences in acclimatisation efficiency was recorded between the different rooting treatments. Plants sporadically started to bloom during

acclimatisation and this was also the case during prolonged *in vitro* cultivation. Furthermore, acclimatisation in non-sterilised substrate failed. However, a fungicide treatment of the substrate could help in this case.

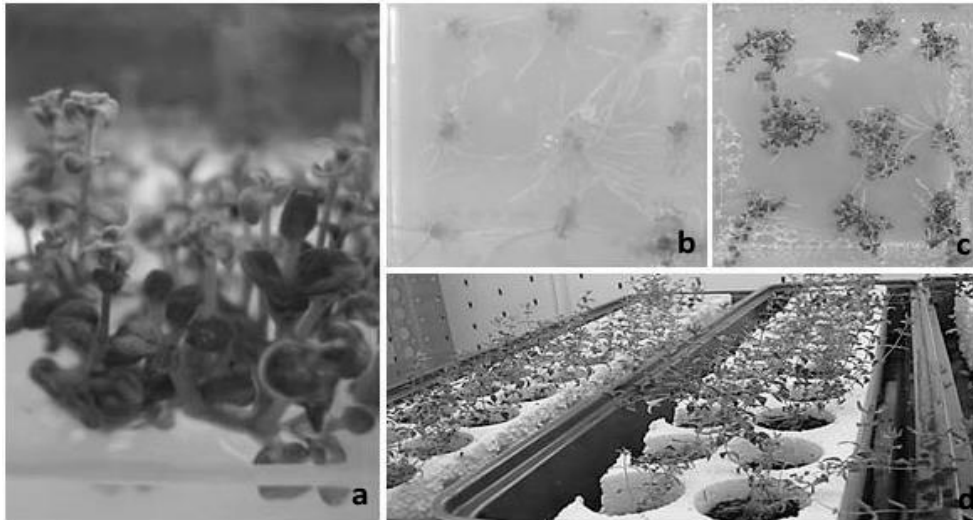


Figure 1. a) Shoot proliferation on PM medium supplemented with 1 mg/L BAP, b) Rooting of *M. croatica* in HFMS, c) Axillary branching during rooting, d) Plants acclimatised in sterile substrate

*Slika 1. a) Proliferacija izdanaka na PM mediju s dodatkom 1 mg/L BAP-a, b) Zakorjenjivanje *M. croatica* u HFMS mediju s agarom, uz dodatak 0.5 mg/L IAA, c) Aksilarno grananje tijekom zakorjenjivanja, d) Biljke aklimatizirane u sterilnome substratu*

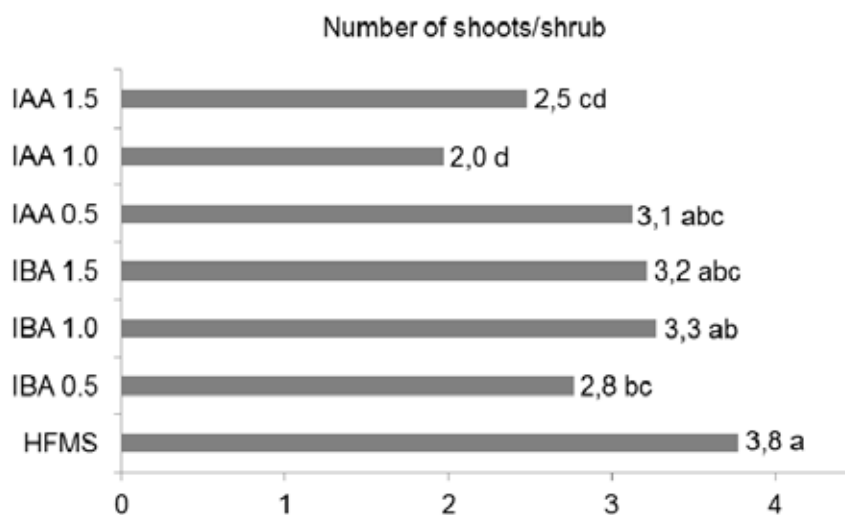


Figure 2. Effect on the number of shoots/shrubs of varying concentrations of auxins (IBA, IAA) during rooting

Slika 2. Utjecaj različitih koncentracija auksina (IBA, IAA) na broj izdanaka po grmiću tijekom zakorjenjivanja

CONCLUSION

In conclusion, an efficient protocol for the micropropagation by axillary branching from adult plant specimen and rooting in agar-based medium was established for *M. croatica*. Due to a number of valuable bioactive compounds as well as ornamental perspective, interest

is continually growing for *M. croatica*. In this study it was found out that media supplemented with 1 mg/L and 2 mg/L of BAP had similar micropropagation efficiency. However, the lower BAP concentration should be recommended as the shoots were longer and the chance for somaclonal variation is considered lower.

Additionally, an explant orientation of vertical or horizontal and varying node positions showed equal capability for shoot proliferation. Importantly, 100% efficiency of rooting was obtained in agar-based medium without PGRs as well as in rooting medium supplemented with IBA 0.5-1.5 mg/L and IAA 0.5 mg/L. The longest roots were obtained in RM with 1 mg/L IBA, but this did not have an effect on the acclimatisation efficiency of the explants with a 90% efficiency achieved in a sterile substrate. In the future, rooting using the agar-based hormone-free medium should be used, with shoots continuing to develop by axillary branching during the rooting process. It is an important feature to consider in the commercial production of *M. croatica*.

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UČINKOVITA MIKROPROPAGACIJA I ZAKORJENJIVANJE VRSTE *Micromeria croatica* (PERS.) SCHOTT (*Lamiaceae*)

SAŽETAK

Hrvatska je bresina (*Micromeria croatica*) ugrožena biljna vrsta, s velikim potencijalom uzgoja u medicinske svrhe i kao ukrasna biljka. Za komercijalnu primjenu, nužan je učinkovit protokol za propagaciju. Cilj ovoga rada bio je uspostavljanje učinkovitoga protokola za mikropropagaciju i zakorjenjivanje vrste *M. croatica* upotrebom odrasle biljke kao izvora početnoga materijala. Testiran je velik broj faktora, uključujući utjecaj 6-benzilaminopurina (BAP) i 1-naftalen octene kiseline (NAA) u različitim koncentracijama, kao i utjecaj orijentacije eksplantata i topofizis efekta na proliferaciju izdanaka. Zakorjenjivanje je ispitivano u mediju s agarom, uz dodatak indol-3-maslačne kiseline (IBA) ili indol-3-octene kiseline (IAA) u različitim koncentracijama. Mediji s 1 ili 2 mg/L BAP-a producirali su najveći broj izdanaka/eksplantatu, međutim preporuča se niža koncentracija BAP-a, na kojoj su dobiveni duži izdanci, bez znakova hiperhidracije. Orijeantacija eksplantata i nodiji s različitim pozicija na izdanku pokazali su jednaku sposobnost proliferacije izdanaka. Na mediju s agarom postignuta je 100% uspješnost zakorjenjivanja. Uspješnost aklimatizacije bila je iznad 90%. Zaključno, razvijen je učinkovit protokol za mikropropagaciju i zakorjenjivanje vrste *M. croatica* za moguću buduću proizvodnju.

Ključne riječi: *in vitro* propagacija, *Micromeria croatica*, biljni regulatori rasta, topofizis efekt

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