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ESSENTIAL OILS AS POTENTIAL BIOCONTROL PRODUCTS AGAINST PLANT PATHOGENS AND WEEDS: *IN VITRO* CULTURE APPROACH

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Abstract: Secondary metabolism in plant plays a major role in the survival of the plant in its ecosystem, mediating the interaction of the plant with its environment. Plant bioactive compounds are biosynthesized as a defensive strategy of plants in response to natural perturbations. A number of biological effects have been associated with the main monoterpenoids detected in investigated *Micromeria* spp. and *Clinopodium* spp. essential oils. One alternative for the production of these prospective biocontrol products is *in vitro* plant tissue culture. Our data suggest that the metabolic potential of *in vitro* shoot cultures of selected species can be manipulated by varying in vitro culture conditions.

Key words: essential oil, glandular trichomes, micropropagation, terpenoids

Introduction

The disadvantages associated with the overuse of synthetic pesticides have stirred the need for the alternative pest and weed management options. Although plants with bioactive compounds have been used to manage different crop pests since ancient times, biological control has gained a lot of interest recently, in line with integrated pest management approaches.

Essential oils constitute an important source of biologically active compounds that display insecticidal, antibacterial, fungicidal, nematicidal, herbicidal activities (Marinkovic et al., 2002; Kaur et al., 2010). Wild-growing plants of the selected Lamiaceae species are characterized by the production of high (*Clinopodium pulegium, Clinopodium thymifolium*) to moderate (*Micromeria graeca, Micromeria croatica, Micromeria juliana*) amounts of the essential oils (Tzakou and Couladis, 2001; Marinković et al., 2002; Slavkovska et al. 2005; Stojičić et al., 2016).

Many of the species belonging to the Lamiaceae family are considered aromatic plants due to the presence of glandular trichomes, which have a distinct ability to synthesize, secrete or store large amounts of specialized metabolites. These

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bioactive compounds play a crucial role in mediating the plant–environment interactions, often rendering a commercial value to the plants that produce them (Giuliani et al., 2017). Therefore, the ability to modulate the density and productivity of glandular trichomes in plants would be of great biotechnological interest.

In vitro culture techniques are indispensable for the production of commercially important plant-derived metabolites, especially of rare plant genotypes. Micropropagation of plants under controlled conditions enables the production of large numbers of homogenous plants year-round and represents a well-founded technology platform for the production of plant natural products.

The large scale production of bioactive compounds with possible future applications in crop pest and weed control, through *in vitro* culture, requires a comprehensive understanding of the influence that culture conditions have on metabolite production. Volatile oil production and emission of *in vitro* cultures are affected by environmental factors, micropropagation techniques, nutrient medium composition, plant growth regulators (Predieri and Raparini, 2007). The present study was initiated to propagate selected aromatic plant species that produce bioactive essential oils; to phytochemically examine the influence of different culture conditions on volatile emissions of micropropagated plants; and to investigate the effects of *in vitro* culture on leaf glandular trichomes, the main structures involved in the essential oil production.

Material and methods

Aerial parts of plants at the vegetative stage of development were randomly collected from wild-growing (natural) populations. Voucher specimens, under the acquisition numbers: № 14623 (*Micromeria graeca*), № 6913 (*Micromeria croatica*), № 10850 (*Micromeria juliana*), № 1441 (*Clinopodium thymifolium*), and № 6912 (*Clinopodium pulegium*), were deposited in the herbarium collection of Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš (HMN).

Plant material – Shoots of wild-growing plants, dissected into one-node stem segments bearing two axillary buds, were used to establish *in vitro* cultures. Surface sterilized nodal segments were transferred to Murashige and Skoog (1962) culture medium (MS) supplemented with 3% (w/v) sucrose, and 0.7% (w/v) agar. Shoot multiplication was carried out on the same medium, by routine subculture performed in 4-week intervals, and on MS media supplemented with plant growth regulators (PGRs). Shoot cultures were maintained in a growth chamber under a 16-h photoperiod, with a photon flux density 45 μE m^{-2} s^{-1} provided by cool white fluorescent lamps, at 25 ± 2 °C. Leaf samples for microscopic analyses were obtained from 10-day old cultures.

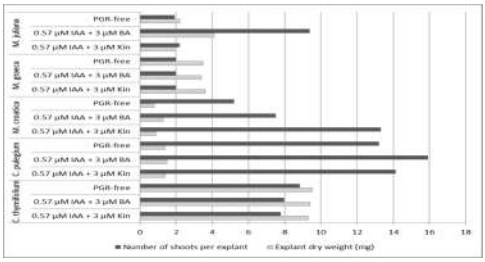
Scanning electron microscopy (SEM) – For SEM analyses, fresh leaves isolated from shoots cultured on MS medium were used. Leaf samples were coated with a thin layer of gold and palladium in a BAL-TEC SCD 005 sputter coater. Both adaxial

and abaxial surfaces were examined with a JEOL JSM-6390 LV (JEOL, Tokyo, Japan) SEM operated at 15 kV.

Light microscopy (LM) – Micromorphological and histochemical analyses were performed on hand-sections of fresh leaves. The following histochemical tests were employed: Sudan IV for total lipids (Jensen, 1962); Nile Blue A for neutral and acidic lipids (Cain, 1947); Nadi reagent for terpenes (David and Carde, 1964). For all the histochemical methods used, control tests were carried out following the suggestions of the respective authors. Sections were examined and photographed using a Zeiss Axiovert light microscope (Carl Zeiss GmbH, Göttingen, Germany).

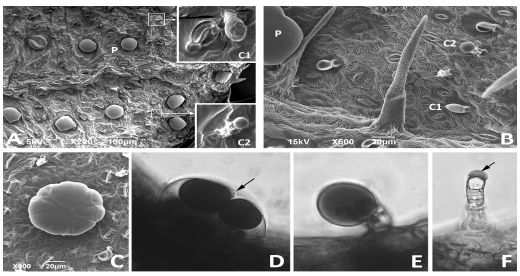
Results and discussion

Nodal segments cultured on MS medium developed axillary shoots. All examined species were successfully propagated on MS medium, while the addition of PGRs affected their multiplication and biomass production in a species-dependent manner (Graph 1).



Graf 1. Efekat biljnih regulatora rastenja i razvića na umnožavanje i rast izdanaka.
Graph 1. Effect of plant growth regulators on shoot proliferation and growth.

SEM and LM analyses showed that leaf indumentum of *in vitro* regenerated plantlets comprised two glandular trichome morphotypes, peltate and capitate, as a characteristic feature of Lamiaceae. Peltate (P) and two submorphotypes of capitate trichomes (C1 and C2) were present on both adaxial and abaxial side, showing an apparently random distribution over the entire leaf surface (Figure 1).



Slika 1. SEM i LM mikrografije žlezdanih trihoma listova odabranih vrsta rodova *Micromeria* i *Clinopodium*, gajenih *in vitro*.

Figure 1. SEM and light micrographs of in vitro leaf trichomes. The abaxial leaf surface of Clinopodium thymifolium (A) and Micromeria croatica (B) showing two types of glandular trichomes, peltate and capitate (insets in A). (C-D) Peltate trichomes of Micromeria graeca at (C) early presecretory-stage and (D) active secretory-stage; note large subcuticular space (arrow) filled with secretory product, stained positive for terpenoids. (E-F) Secretory-stage capitate trichomes: C1, showing unicellular head stained positive for essential oils (E) and C2, showing apical secretory cell with large subcuticular space (arrow) filled with secretion (F).

Tabela 1. Histohemijska karakterizacija sekretornih produkata žlezdanih trihoma lista odabranih vrsta rodova *Micromeria* i *Clinopodium*

Table 1. Histochemical characterization of secretions of leaf glandular trichomes of selected Micromeria and Clinopodium species

Chemical compounds/ hemijska jedinjenja	*Micromeria graeca*			*Micromeria croatica*			*Clinopodium thymifolium*			*Clinopodium pulegium*		
	P	C1	C2	P	C1	C2	P	C1	C2	P	C1	C2
Lipids/masti	++	+–	+	++	+–	+	++	+	+–	+	+	++
Essential oils/esencialna ulja	+	+–	+	+	+–	+	++	+	+-	++	+	++
Terpenoids/ terpenoidi	++	+–	+	++	+–	+	++	+	+-	+	+	++

–negative; +–slightly positive; +positive; ++strongly positive

* P – peltate trichome; C1 - Type I capitate trichome; C2 - Type II capitate trichome

Terpenoids account for the largest share of the volatile organic compounds in the majority of examined *Micromeria* and *Clinopodium* species (Marinković et al., 2002; Slavkovska et al., 2005; Tošić et al., 2019). The ecological role in defense against diverse environmental challenges has been suggested for the main compounds detected in their essential oils, such as menthone, pulegone; piperitone, piperitenone and their oxides (Economou and Nahrstedt, 1991; Mucciarelli et al., 2001). Besides deterring herbivores and inhibiting microbial growth, monoterpenoids have been recognized as the main allelochemicals in higher plants. This is most likely correlated with their respiration-inhibitory activity, with pulegone and menthone being the most effective in inhibition of plant respiratory functions (Mucciarelli et al., 2001).

A number of pharmacological and biological effects that have been associated with the main monoterpenoids detected in selected *Micromeria* spp. and *Clinopodium* spp. essential oils, paves the way to their possible future applications in crop pest and weed control. The morphogenetic and metabolic potential of plants in micropropagation systems can be greatly manipulated by varying *in vitro* culture conditions. Therefore, our data suggest that *in vitro* shoot cultures of the selected species could be an advantageous source for obtaining these valuable metabolites throughout the year.

Conclusion

Our results suggest that the careful selection of the culture conditions could increase the accumulation of biomass and the production of secondary metabolites of selected *Micromeria* and *Clinopodium* plant species, which could be employed to obtain essential oils of relatively stable composition for commercial use.

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ETERIČNA ULJA KAO POTENCIJALNI BIOKONTROLNI PROIZVODI PROTIV BILJNIH PATOGENA I KOROVA: U *IN VITRO* KULTURI

Branka Uzelac[1], Dragana Stojičić[2], Snežana Budimir[1], Svetlana Tošić[2], Bojan Zlatković[2], Saša Blagojević[2], Branislav Manić[2], Mirjana Janjanin[1], Violeta Slavkovska[3]

Izvod

Sekundarni metabolizam igra glavnu ulogu u opstanku biljke u njenom ekosistemu, posredujući u interakciji biljke sa svojom okolinom. Biljna bioaktivna jedinjenja su biosintetizovana kao odbrambena strategija i kao odgovor na prirodne perturbacije. Brojni biološki efekti su povezani sa glavnim monoterpenoidima otkrivenim u esencijalnim uljima *Micromeria* spp. i *Clinopodium* spp.. Jedna od alternativa za proizvodnju ovih perspektivnih biokontrolnih proizvoda je u *in vitro* kulturi biljnog tkiva. Naši podaci sugerišu da se metaboličkim potencijalom u *in vitro* praćenju kultura izabranih vrsta može manipulisati različitim uslovima *in vitro* kulture.

Ključne reči: esencijalna ulja, žlezdani trihomi, mikropropagacija, terpenoidi

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