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ASSESSMENT OF THE EFFECT OF PLANT GROWTH REGULATORS ON *IN VITRO* MICROPROPAGATION AND METABOLIC PROFILES OF *MELISSA OFFICINALIS* L. (LEMON BALM)

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ABSTRACT

In this study the effectiveness of plant growth regulators on micropropagation, total phenol content and metabolic profiles of *Melissa officinalis* L., important medicinal herb was assessed. The stem segments derived from one-month old *in vitro* germinated seedlings were used for initial explants. Of the eight different nutrient media tested the most favorable for micropropagation were found to be MP2 (MS medium enriched with 1mg/L BAP and 0.1 mg/L IBA) and MP3 (MS supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA), which yielded 3.25- 4.0 shoots per explant within 4 weeks of culture. *In vitro* rooting (100%) was achieved on half strength MS medium contained 2% sucrose. Rooted plantlets were adapted successfully (98%) in a mixture of soil, peat, perlite, and sand in ratio 2:1:1:1 (v/v/v/v). Twenty metabolites were identified by GC/MS analysis of shoots grown on the MS media including different types of growth regulators. Analysis revealed the presence of phenolic, organic and fatty acids, sterols, triterpenes, fatty alcohols, saccharides and polyols. The studied samples have the same metabolic profile with quantitative differences in the individual metabolites. The maximum content of total phenols (13.92 mg/g extract) was obtained in shoots grown on MP2 medium and the lowest was recorded in the microplants cultured on MP1 control medium (8.45 mg/g extract). The obtained results indicated that it is possible to achieve the accumulation of desired metabolites by selection of plant growth regulators added in nutrient media.

Keywords: *in vitro*, multiplication, total phenols, metabolites, GC/MS analysis, medicinal plant

INTRODUCTION

Melissa officinalis L. known as Lemon Balm is an aromatic medicinal plant species of the family Lamiaceae spread from the Central and Southern Europe to Iran and Central Asia. *M. officinalis* plants are a rich source of various secondary metabolites – phenolic acids (rosmarinic acid, caffeic acid, chlorogenic acid, protocatechuic acid), flavonoids (luteolin, apigenin etc.), monotrpenes and sesquiterpenes, volatile oils and tannins **(Carnat *et al.*, 1998; Adinee *et al.*, 2008; Moradkhani *et al.*, 2010; Shakeri *et al.*, 2016)**, which possess sedative, antibacterial, anti-inflammatory, neuroprotective, antivirus and antioxidant effects **(López *et al.*, 2009; Bounihi *et al.*, 2013; Kamdem *et al.*, 2013; Denzler *et al.*, 2016; Alizadeh Behbahani and Shahidi, 2019; Salamon *et al.* 2019)**. The aerial part of plant is used for treatment of neurodegenerative diseases, insomnia, gastrointestinal and cardiac disorders etc. **(Scholey *et al.*, 2014; Shakeri *et al.*, 2016)**. The cultivation of lemon balm through conventional methods is not difficult, but the main problem is to obtain a homozygous population producing valuable biologically active substances with constant quality **(Meftahizade *et al.*, 2010)**. Through *in vitro* techniques it is possible to achieve both stable quantity and increase of the content of desired metabolites in plant culture **(García-Pérez *et al.*, 2011)**. Micropropagation allows true-to-type multiplication of a selected genotype **(Debergh and Read, 1991)**. The differentiated organ cultures like shoots and roots cultured *in vitro* can present a metabolite profile similar to that of initial plants **(Kolewe *et al.*, 2008)**, maintaining the same genetic characteristics of the highest productive clones **(Chaturvedi *et al.*, 2007)**. The various parts of lemon balm plants (shoots tips, cotyeldonary nodes, hypocotyeldon axes, leaf, petiole, stem, axillary buds) derived from seedlings or field grown plants were used as explants to initiate *in vitro* cultures **(Tavares *et al.*, 1996, Silva *et al.*, 2005; Meftahizade *et al.*, 2010; Ülgen *et al.*, 2020)**. Morphogenesis response of *M. officinalis* was affected by genotypes, initial explants, type of plant growth regulators (PGRs) etc. **(Meftahizade *et al.*, 2010; Ülgen *et al.*, 2020)**. The manipulation with PGRs (type, concentration and combination of them) is a simple tool for regulation of growth of shoots under *in vitro* conditions and the different branches of secondary metabolism in plant cultures **(Matkowski, 2008; Smetanska, 2008; Luczkiewicz *et al.*, 2014)**. In recent years the studies related to improvement of the production of certain compounds by exploring plant tissue culture raised great interest **(Dias *et al.*, 2016)**. However the research connected with accumulation of secondary metabolites in *in vitro* culture of *M. officinalis* is limited **(Barros *et al.*, 2013; Mokhtarzadeh *et al.*, 2016, Mousavi and Shabani, 2019)**.
The objective of this work was to study the effect of different types of plant growth regulators on micropropagation, total phenol contents and metabolic profiles of *in vitro* propagated *M. officinalis* shoots.

MATERIALS AND METHODS

Sterilization

The seeds were collected from *ex situ* collection of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. Seeds were surface washed with tap water and a detergent for 20 min followed by soaking in 70% ethanol for 2 min. Then two sterilizing agents were tested: 1) treatment with 50% commercial bleach (ACE) containing 4.85% sodium hypochlorite with a drop of Tween 20 or 2) 0.1% $HgCl_2$ (Merck, Germany) each applied for 10 min. The sterilized seeds were washed three times with sterile distilled water for 5, 10 and 15 min.

Seed germination

Sterilized seeds were germinated in Petri dishes containing three variants **Murashige and Skoog, (1962)** (MS)-based medium, 30 seeds per variant (Table 1): 1) MSG1 control (without PGR), 2) MSG2 supplemented with 5 mg/L gibberellic acid (GA_3), 3) MSG3 containing 10 mg/L GA_3. All media contained 2% sucrose and were solidified with 0.7% agar (w/v).

Micropropagation

Stem segments (excised from one-month seedlings) were used as initial explants for multiplication. They were cultured on full-strength MS medium supplemented with one of the following cytokinins: 6-benzylaminopurine (BAP), kinetin (Kin), zeatin (Zea) or N^6[2-isopentenyl]-adenine (2-iP), combined with the auxins: indole-3-butyric acid (IBA) or α-naphthalene acetic acid (NAA). The media contained 3% sucrose and were solidified with 0.7% agar (w/v). The concentration and combinations of PGRs are shown in Table 2. Initiation of shoots and micropropagation experiments were carried out using glass tubes, 10 mL medium per tube. At the end of 4 weeks culture period, the frequency of shoot proliferation (evaluated as % of explants forming shoots), the multiplication rate (evaluated as mean number of shoots (>1 cm) per explant), and the mean shoot height were recorded. The hyperhydricity (%) and the rooting (%) observed in some variants were estimated too. Each treatment consisted of 20 explants distributed in 2 replications. Two subsequent sub-cultivations were done.

***In vitro* rooting and acclimatization of *ex vitro* obtained plants**

For induction of *in vitro* rhizogenesis stem explants consisting of one node (1.5 - 2 cm) were transferred to half strength MS medium containing 2 % sucrose. The *in vitro* obtained plants with well-developed roots were removed from culture vessels and washed free of agar. They were transferred to small pots containing soil, peat, perlite, and sand in ratio 2:1:1:1 (v/v/v/v). The pots were covered with clear plastic box to maintain high relative humidity and plants were watered daily. After two weeks the plastic boxes were removed. The survival percentage of plants was assessed after 4 weeks. Then the *ex vitro* adapted plantlets were transferred to the experimental field for further acclimation.

Culture conditions

All media were adjusted to pH 5.7 with 1N NaOH before autoclaving. The nutrient media were autoclaved at 120 ºC for 20 min at 1atm. The cultures were maintained in a growth chamber under a 16h day/8h dark photoperiod provided by cool-white fluorescent lamps, irradiance 40 µmol $m^{-2}s^{-1}$ at temperature 21±2 °C.

Preparation of extracts and GC/MS analysis

Six samples were subjected to GC/MS analysis, each consisting of 100 mg dried *in vitro* shoots grown on MP1 control, MP2, MP4, MP5, MP7 and MP8 media. Samples were extracted with methanol by maceration for 24 hours at room temperature with added internal standard 3,4 dichloro-4-hydroxybenzoic acid (50 µg/mL) at the beginning of the extraction. The amounts of metabolites were estimated against this standard. For GC/MS analysis 300 µL of each extract was transferred to a vial and evaporated to dryness, then silylated with 50 µL of N, o-bis- (trimethylsilyl) trifluoroacetamide (BSTFA) in 50 µL of pyridine for 2 h at 50 °C. The spectra were recorded on a Thermo Scientific Focus GC combined with a Termo Scientific DSQ mass detector as described previously (**Nikolova *et al.*, 2019**). The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds was recorded with standard n-hydrocarbon calibration mixture (C9-C36).

Total phenolic content

The total phenol content was determined spectrophotometrically using *Folin–*Ciocâlteu *reagent* and was expressed as mg per gram of the plant extract, in gallic acid equivalents (GAE) (**Nikolova *et al.*, 2013**).

Statistical analysis

Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the 5% significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows). Data were presented as means ± standard deviation.

RESULTS

Seed germination and establishment of *in vitro* culture

The surface sterilization with bleach was successful in ensuring contamination free seeds. Application of the mercuric chloride also resulted in 100% sterilized seeds, but they subsequently did not germinate on any of the tested nutrient media, indicating that the exposure time of this sterilizing agent was exceeded. Ninety percent of seeds germinated on MSG1 control medium free of plant growth regulators (Table1). The seeds cultured on MSG2 and MSG3 showed lower percentage of germinated seeds, 40% and 20%, respectively, which suggested a surplus of the gibberellic acid used.

Table 1 Seed germination of *M.officinalis* on different nutrient media

MS medium variant (supplements in mg/L)	Cultured seeds (number)	Germinated seeds %
MSG1 (control medium)	30	90
MSG2 (5 mg/L GA_3)	30	40
MSG3 (10 mg/L GA_3)	30	20

Table 2 Effect of the PGRs (mg/L) on the micropropagation of *M. officinalis*

Abbr.	BAP	Kin	Zea	2-iP	IBA	NAA	Shoot proliferati on %	Multiplication rate	Shoot height cm	Features
MP1 control							15	1.15±0.37^a	7.49±3.33^f	roots (90%)
MP2	1				0.1		80	3.25±1.07^c	2.46±0.92^{ab}	-
MP3	1.5					0.5	90	4.00±1.21^d	1.91±0.95^{ab}	hyperhydricity (15%)
MP4	2				0.1		90	4.30±1.34^d	1.83±0.78^a	hyperhydricity (60%)
MP5		1			0.1		70	2.60±1.14^{bc}	3.62±1.39^{cd}	-
MP6		1.5				0.5	75	2.80±1.36^c	2.95±1.11^{bc}	-
MP7			1		0.1		65	1.95±0.88^b	4.58±1.44^d	roots (20%)
MP8				1	0.1		20	1.20±0.41^a	6.05±2.08^e	roots (70%)

The data are presented as means of 20 shoots per medium variant ± standard deviation. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one way ANOVA analysis.

Influence of plant growth regulators on micropropagation

The effectiveness of micropropagation depended on the nutrient media composition (Table 2). The explants cultured on control medium (MP1 free of PGRs) showed high shoot elongation, but only 15% of them produced 1-2 shoots per explants. The use of nutrient media supplemented with BAP and auxin (MP2, MP3 and MP4) resulted in maximum proliferation frequency (80 and 90%). Concerning the quantity of the obtained shoots, the combination of BAP (1.5 mg/L) and NAA (0.5 mg/L) was optimum for lemon balm micropropagation and an average of 4.0 new shoots per explant within 4 weeks were formed (Figure 1a); however, both MP3 and MP2 were commensurable because of the hyperhydricity (15%) observed in MP3. Increased concentration of BAP up to 2 mg/L (MP4) led to increase of multiplication rate (4.30±1.34), but most of the plants (60%) suffered from hyperhydricity. The nutrient media containing kinetin (MP5 and MP6) were appropriate for obtaining of vigorous shoots and the mean numbers of shoots per explant were 2.60±1.14 and 2.80±1.36, respectively. Media supplemented with Zea or 2-iP expressed less shoot proliferation and lower multiplication rate, compared with those containing BAP or Kin (Table 2). The shoots produced on BAP or Kin containing media were characterized by very short internodes, small leaves and small shoot height in comparison with those grown on the media fortified with 2-iP or Zea, which were big, with longer internodes, and large leaf area.

In second subculture the multiplication rate was maintained and even increased in the optimal nutrient medium MP3 (4.50±1.44) (Figure 1b). However in subsequent subcultures there were noticed decrease of multiplication rate and regenerative capacity of *M. officinalis*. The shoots grew in height (8-12 cm) in short period of

time (4 weeks) on all tested media and were cut into 3-4 nodal segments and again sub-cultured on fresh media. Thus, a large number of new shoots was obtained.

Rooting and acclimatization of *in vitro* obtained plants

Roots appeared during the first 5-6 days of culture on half strength MS medium. All *in vitro* shoots (100%) developed roots with an average number of 5-7 roots per shoot (Figure 1c). The 2:1:1:1 ratio of soil, peat, perlite and sand was found to be optimum for the hardening of the plants (the survival rate of 98%). This soil mixture had a beneficial effect on plant growth and development (Figure 1d). No phenotypic variations in the *ex vitro* adapted plants were observed.

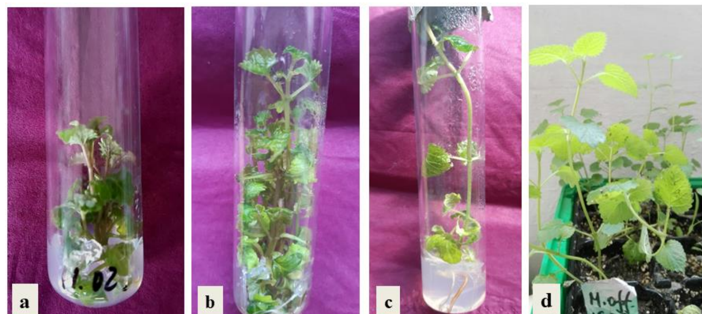

Figure 1 *In vitro* cultivation of *M. officinalis*: Micropropagation on MP3 medium (MS supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA: a) first subculture) and b) second subculture; c) *In vitro* rooting on ½ MS nutrient medium; d) *Ex vitro* adapted plants

Influence of plant growth regulators on metabolite profiles and total phenolic content

The applied plant growth regulators in nutrient media influenced not only micropropagation but also the accumulation of metabolites in *in vitro* obtained shoots. The results of GC/MS analysis of the metabolic profiles of the shoots grown on media containing 0.1 mg/L IBA and 4 different cytokinins in concentration 1 mg/L, are presented in Table 3. Twenty metabolites were identified. The studied samples have the same metabolic profile with quantitative differences in the individual metabolites. The phenolic acids – caffeic and rosmarinic were found to be accumulated in the highest amount in shoots grown on MS media containing BAP (MP2 and MP4). The organic acids were established in the highest quantity in the shoots cultured on MP7 containing Zea. At the same cultural conditions increased accumulation of sterols, triterpenes and fatty acids, as well as glycerol was observed. Also the fatty acids were presented in high content in the shoots grown on MP8 supplemented with 2-iP. In these cultural conditions the myo-inositol was accumulated in the highest degree. The content of saccharides was found to increase in all tested nutrient media containing PGRs compared to the control medium. A reverse trend was noticed for the content of pyroglutamic acid, which was significantly higher in shoots from the control medium.

Table 3 Identified compounds of the studied samples

Compounds	RI	Samples					
		MP1	MP2	MP4	MP5	MP7	MP8
Phenolic, organic and fatty acids							
Caffeic acid	2130	0,09	0,36	0,23	0,05	0,15	0,19
Rosmarinic acid	3739	0,32	1,74	2,63	0,79	0,53	1,01
Succinic acid	1305	0,02	1,60	0,99	1,89	2,85	1,62
Glyceric acid	1319	0,13	0,10	0,15	0,09	0,18	0,06
Malic acid	1474	0,10	0,23	0,21	0,27	0,33	0,10
Pyroglutamic acid	1515	48,54	5,05	5,45	4,97	2,84	7,43
Octanoic acid	1523	0,28	0,38	0,18	0,26	0,37	0,50
Hexedecanoic acid (C16:0)	1924	0,05	0,15	0,11	0,27	0,37	0,34
Octadecadienoic acid (C18:2)	2091	0,00	0,02	0,00	0,03	0,06	0,07
Octadecatrienoic acid (C18:3)	2097	0,03	0,10	0,10	0,16	0,29	0,29
Sterols and Triterpenes							
ß-Sitosterol	3338	0,72	0,69	0,85	0,82	1,28	0,23
Triterpene acids	3854	0,02	0,14	0,03	0,04	0,04	0,01
Saccharides and Polyols							
Glycerol	1260	3,23	4,49	1,68	5,05	8,68	5,87
Fructose 1	1790	6,34	3,67	22,70	3,88	14,50	4,48
Fructose 2	1806	16,20	19,18	24,15	5,30	17,79	1,21
Fructose 3	1812	3,95	5,51	6,91	0,17	1,20	0,68
Glucose	1889	1,08	18,78	2,33	12,67	6,20	32,71
Monosaccharide	1982	5,96	9,64	3,50	5,69	17,21	5,70
Myo-Inositol	2084	1,47	0,53	0,38	0,98	1,57	1,83
Sucrose	2629	11,69	27,15	28,34	57,55	25,96	36,12

The results of total phenolic determination are presented at Figure 2. It was found that shoots from variants with higher micropropagation efficiency such as BAP and Kin containing media, produced an increase amount of total phenols in comparison with control media MS free of PGRs. The maximum amount of total phenols (13.92 mg/g extract) was obtained in shoots grown on MP2 (MS medium enriched with 1 mg/L BAP and 0.1 mg/L IBA) and the lowest one was recorded in the microplants cultured on MS control medium (8.45 mg/g extract).

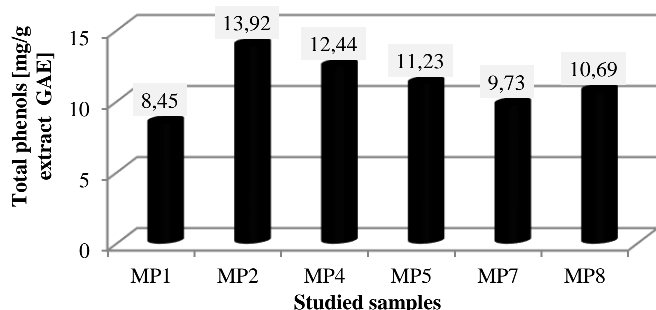

Figure 2 Total phenolic content in the studied samples

DISCUSSION

In recent years, scientists have become increasingly interested in accumulation of secondary metabolites in plants cultured under *in vitro* conditions. Plant tissue cultures were used as a model system for studying regeneration capacity of plants and biosynthesis of compounds with pharmaceutical properties **(Chandran *et al.*, 2020)**. It have been reported that PGRs affected developmental process and secondary metabolites production **(Palacio *et al.*, 2008; Tasheva *et al.*, 2012; Baskaran *et al.*, 2012)**. The growth of plants in *in vitro* conditions is influenced by the genotype, endogenous concentration of PGRs and response of tissue were related to the interaction between exogenous PGRs and their receptors **(Phillips and Garda, 2019).** In this study an optimized protocol for *M. officinalis* micropropagation as well as metabolitic profile and total phenol content of *in vitro* grown shoots were presented. Surface sterilization (100% efficiency) was attained by consecutive soaking the seeds in 70% ethanol for 2 min and in bleach solution for 10 min. The use of 2.5% sodium hypochlorite for 3 minutes was effective disinfectant for obtaining of lemon balm *in vitro* aseptic seeds **(Kiani *et al.*, 2017)**. Other authors recommended applying of more toxic sterilizing agent $HgCl_2$ (0.1%) for 15 minutes, but they did not mention the disinfection efficiency **(Ülgen *et al.*, 2017)**. In our study the sterilization procedure with 0.1% $HgCl_2$ failed to induce germination of seeds on all nutrient media tested. The highest germination efficiency of seeds was achieved on MS nutrient medium free of PGRs (90%) and

lower was recorded on MS media supplemented with GA_3. Although GA_3 usually stimulated seed germination in some cases it is suppressed by applying inappropriate concentrations (**Chetouani *et al.*, 2017**). In literature there are many trials to stimulate *M. officinalis* seed germination: using of magnetic field application (100 mT) for 1 hour resulted in 52% germinated seeds (**Ülgen *et al.*, 2017**), pre-chilling treatment lead to 68% germination (**Aghilian *et al.*, 2014**), incubation at 25 ºC temperature was optimum for 93% seeds germination (**Vârban *et al.*, 2010**). **Kiani *et al.* (2017)** reported that the most appropriate medium for *in vitro* germination (81.33%) was half strength MS medium containing 1% sucrose; however in our study we achieved 90% germination on full strength MS medium with 2 % sucrose. The most favorable media among the tested eight variants were found to be those supplemented with 1.5 mg/L BAP and 0.5 mg/L NAA, or with 1 mg/L BAP and 0.1 mg/L IBA. Most authors recommended addition of BAP at concentration of 1 to 3 mg/L in combination with auxins NAA or IAA to nutrient medium MS for *M. officinalis* shoot multiplication using different type of initial explants (shoot tips, apical nodes, cotyledonary nodes) (**Tavares *et al.*, 1996, Mészáros *et al.*, 1999; Gheorghita *et al.*, 2005; Meftahizade *et al.*, 2010**), however, our results showed hyperhydricity when BAP was in concentration higher than 1 mg/L. The authors observed an inverse relationship between the number of produced shoots and their elongation (**Tavares *et al.*, 1996; Meftahizade *et al.*, 2010**), which has been confirmed also in our research. Efficiency of BAP for shoots multiplication may be related to the ability of plant tissue to metabolize BAP more easily than other synthetic growth regulators, or to synthesize natural plant hormones (such as zeatin) in presence of BAP. (**Malik *et al.*, 2005**). Best response regarding regeneration of lemon balm was observed using shoot tips compared with leaf segments (**Meftahizade *et al.*, 2010**). **Aasim *et al.* (2018)** reported maximum adventitious shoot regeneration of cotyledonary leaf explants of *M.officinalis* cultured on MS media supplemented with 0.4 mg/L TDZ and 0.1 mg/L IBA with additional 1 mg/L polyvinylpyrrolidine. In our study 100% root formation of shoots was obtained on low-cost and simple medium (half strength MS medium, containing 2 % sucrose) without PGRs. According to the other authors for *in vitro* root induction of *M. officinalis* auxins (NAA or IBA) need to be added to the nutrient media (**Tavares *et al.*, 1996; Meftahizade *et al.*, 2010**). The obtained *in vitro* micropropagated shoots were subjected for GC/MS analysis. The received results showed that the amounts of the individual metabolic groups vary depending on the cultural conditions (in particular the composition of the nutrient media). By selection of PGRs in nutrient medium it is possible to achieve the accumulation of desired metabolites**.** In addition the obtained differences could be interpreted, from the different stages of plant development. The shoots grown on MP1 (control medium) and 2-iP containing media (MP8) were fully developed in plants and were characterized by the formation of roots, while those cultured on the other media did not develop roots. Of the four fatty acids identified, saturated fatty acids (octanoic acid and hexedecanoic acid) were presented in a larger amount than unsaturated (octadecadienoic acid and octadecatrienoic acid). This is in agreement with results obtained by **Abdel-Naime *et al.* (2019)**, who studied petroleum ether fraction of *M. officinalis* by GC/MS and identified fatty acids (mainly saturated) as the most abundant acids was palmitic acid. In addition the authors established varied terpenoids, especially triterpenes. The growth regulators (IAA and BAP) influenced essential oils composition of *M. officinalis* by promoting the delay of the alcohol oxidation to aldehydes (**Silva *et al.*, 2005**). The treatment with abscisic acid (ABA) was found to increase endogenous ABA content and rosmarinic acid synthesis in the shoot cultures of *M. officinalis* (**Mousavi and Shabani, 2019**). The growth regulators such as BAP and meta-topolin (mT) activated secondary metabolites production in medicinal plant species through stimulation of biosynthetic pathway (**Aremu *et al.*, 2012; Coste *et al.*, 2011**). It was noticed that BAP enhanced the accumulation of hydroxybenzoic derivatives while topolins increased levels of hydroxycinnamic acid derivatives in *Merwilla plumbea* (**Aremu *et al.*, 2013**). The nutrient media MS supplemented with BAP and IBA led to higher activity of antioxidant enzymes in micropropageted *Hyssopus officinalis* L. shoots in comparison with those cultured on nutrient media with TDZ or Zea (**Zayova *et al.*, 2018).** It was found that plant growth regulators initiate or mediate many different physiological processes and interact with each other in various ways during these processes (**Phillips and Garda, 2019).** Future research should be done in order to clarify the role of specific growth regulators in the accumulation of corresponding metabolites.

CONCLUSION

The results obtained from this study pointed that the type and concentration of plant growth regulators affected not only micropropagation and growth of shoots *in vitro*, but also the quantity of total phenols and the individual metabolites of *Melissa officinalis* L. However, all samples showed the same metabolic profile assessed by GC/MS analysis. In addition an optimized protocol for micropropagation of *M. officinalis* was presented, allowing 98% *ex vitro* adapted plants. The results reveal that lemon balm *in vitro* plants may offer a promising source of desired substances of this important medicinal plant.

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