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

Maria Petrova*1, Milena Nikolova2, Margarita Dimitrova1, Lyudmila Dimitrova1

Address(es):
1Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bldg. 21, 1113 Sofia, Bulgaria.
2Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bldg. 23, 1113 Sofia, Bulgaria.

*Corresponding author: marv_petrova@yahoo.com
https://doi.org/10.15414/jmbfs.4077

ARTICLE INFO
Received 13. 12. 2020
Revised 9. 6. 2021
Accepted 16. 6. 2021
Published 1. 12. 2021

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 containing 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.), monoterpens 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 homogenous population producing valuable biologically active substances with constant quality (Meftahizade et al., 2010). 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 (Merck, Germany) each applied for 10 min. The sterilized seeds were washed three times with sterile distilled water for 5, 10 and 15 min. The 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 (GA3), 3) MSG3 containing 10 mg/L GA3. 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\(^\text{\textregistered}\)-2-isopentenyl-adenine (2-iP), combined with the auxins: indole-3-butyric acid (IBA) or 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 °C for 20 min at 1atm. The culture vessels 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 Thermo 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–Ciúccie 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 2** 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)                       | 30                      | 40                 |
| MSG3 (10 mg/L GA)                      | 30                      | 20                 |

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 (4.30±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.

The results of total phenolic determination on 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 medium 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 pyrogallinic acid, which was significantly higher in shoots from the control medium.

### DISCUSSION

In recent years, scientists have become increasingly interested in accumulation of secondary metabolites in plants cultured under in vitro conditions. In this study, the use of a healthy and efficient method for the disinfection of lemon balm leaves and shoots under optimal 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 phenolic content of shoots grown on different cultural conditions was compared to the control medium. A reverse trend was noticed for the content of pyrogallinic acid, which was significantly higher in shoots from the control medium.

Table 3 Identified compounds of the studied samples

| Compounds                      | RI  | 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 |
| Pyrogallinic 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 |
| Hexadecanoic acid (C16:0)      | 1924| 0.05 | 0.15 | 0.11 | 0.27 | 0.37 | 0.34 |
| Octadecanoic acid (C18:2)      | 2091| 0.00 | 0.02 | 0.00 | 0.03 | 0.06 | 0.07 |
| Octadecatetraenoic acid (C18:3)| 2097| 0.03 | 0.10 | 0.10 | 0.16 | 0.29 | 0.29 |
| Sterols and Triterpenes        |      |      |      |      |      |      |      |
| β-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 Polysaccharides|      |      |      |      |      |      |      |
| 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 | 0.38 | 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
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. In addition, 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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