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ARTICLE

Growth, secondary metabolites production, antioxidative and antimicrobial activity of mint under the influence of plant growth regulators

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ABSTRACT The effects of plant growth regulators on *Mentha piperita* explants cultured *in vitro* were studied for the purpose of analyse growth, secondary metabolite production, antioxidant and antimicrobial activities in micropropagated plants. The basal medium was experimentally supplemented with the auxin, indole-3-butyric acid (IBA) and the cytokinin, N6-benzyladenine (BAP) individually and in combination. Treatment with BAP and IBA resulted in an increased shoot and root number. The production of phenolic compounds was affected by the addition of the highest concentration of BAP, while antioxidant and antimicrobial activities were affected by several BAP and IBA treatments. Our results demonstrate that the application of growth regulators increases growth and secondary metabolite productions in the medicinal herb *M. piperita*.

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Introduction

Oxygen free radicals induce damage due to membrane peroxidation, which leads to tissue damage. The consequences include peroxidation of lipids, oxidation of proteins, enzyme inhibition, activation of programmed cell death pathway and finally to death of the cells (Mittler 2002). In order to minimize damage induced by free radicals, plants have a number of mechanisms, such as the antioxidants. Many aromatic and medicinal plants contain chemical compounds showing antioxidant properties (Gautam et al. 2012). The most abundant is family Lamiaceae with numerous species that are used as spice and medicinal herb. One of them is mint (*Mentha piperita* L.) that is used in the traditional medicine to relieve the symptoms of vomiting, indigestion, stomach and menstrual cramps and parasitosis (Baliga and Rao 2010; Kavina et al. 2011). It is also known for its carminative, stimulant, antispasmodic, antiseptic, anti-inflammatory, antibacterial and antifungal activities (Guedon and Pasquier 1994; Sean et al. 2004).

Flavonoids and phenolic acids are the major classes of phenolic compounds, whose antioxidant activity is described in a number, of papers (Nenadis et al. 2004). In addition to antioxidant activity, many phenolic compounds have been shown to exert anticancer or antimutagenic activity (Tapiero et al. 2002; Awale et al. 2005). *In vitro* culture techniques today present an effective alternative tool for the production, of secondary metabolites, with even higher secondary metabolites production than the intact plants (Parr 1989; Rao and Ravishankar 2002). Plant growth regulators are crucial factors in growth and secondary metabolite biosynthesis in plant tissue cultures. For example, they noticed that the auxins and cytokinins influences both growth index and rosmarinic acid accumulation in *Coleus blumei* (Qian et al. 2009) or cardiac glycosides production in *Digitalis lanata* (Palazon et al. 1994). Alterations in the type and concentration of auxin or cytokinin as well as the auxin/cytokinin ratio have strong effects on both growth and metabolite formation in plants (Rao and Ravishankar 2002). Auxin/cytokinin ratio appears to be the primary factor controlling growth and morphology, while the effects on secondary metabolite formation varied and depended on plant species (Sharafzadeh and Zare 2001; Scravoni et al. 2006). The effects of some plant growth regulators on secondary metabolite production have been already studied and confirmed (Shukla and Farooqi 1990; Stoeva and Iliev 1997; Sharafzadeh and Zare 2001; Farooqi et al. 2003; Arikat et al. 2004).

The aim of this study was to analyze the effect of different concentration of a cytokinin, N6-benzyladenine purine (BAP) alone or in combination with an auxin, indole-3-butyric acid

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(IBA) on the growth, biochemical changes and secondary metabolites production of *Mentha piperita* L. cultures *in vitro*.

Materials and Methods

Plant materials and treatments

Commercially purchased seeds of *M. piperita* L. (Sjemenarna, Ljubljana, Slovenia) were surface-sterilized and germinated on a MS basal medium (Murashige and Skoog 1962). Shoot multiplication was investigated by culturing epicotyls at different level (0.0, 0.1, 0.5, 1.0, 2.0 and 4.0 mg/L) of N6-benzyladenine (BAP) alone (BH; B0.1; B0.5; B1; B2; B4) or in combination with 0.1 mg/L indole-3-butyric acid (IBA; BI0.1; BI0.5; BI1; BI2; BI4). The pH of all media was adjusted to 5.8 with 1 M KOH or 1 M HCl before autoclaving at 121 °C for 20 min. All cultures were incubated at 24 ± 1 °C under cool white fluorescent lights (40 μmol $m^{-2}s^{-1}$) and with the 16 h light photoperiod. The plants were collected on thirty days after treatment and used for the analysis of growth parameters, photosynthetic pigments and secondary metabolite content, antioxidant and antimicrobial activity.

Growth parameters and photosynthetic pigments

Number of shoots and roots per explant was recorded after 30 days of cultivation. Extraction of photosynthetic pigments followed the method described by Porra et al. (1989). Quantification was done by spectrophotometric determination of the absorbance at 663 nm (chlorophyll a), 646 nm (chlorophyll b) and 440 nm (carotenoids) according to Porra et al. (1989) and Holm (1954). The concentrations of photosynthetic pigments were expressed as mg of pigments per g fresh weight (mg/g FW).

Secondary metabolite analysis

Sample preparation

Aerial parts of *in vitro* cultivated mint were air dried and grounded in a mixer. A portion of the finely powdered material was extracted three times with 70% methanol during a 24 h period at 4 °C. After removal of methanol all extracts were evaporated to dryness and then dissolved in absolute ethanol to make 1% (w/v) solutions.

Determination of total phenol content

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Pal et al. 2009). Phenols were determined by spectrophotometric readings at 765 nm. The standard curve was prepared using catechine as standard. Total phenol values are expressed in terms of catechine equivalent (mg/g of dry mass - DM).

Determination of total flavonoid content

Colorimetric aluminium chloride method was used for flavonoid determination (Waterhouse 2001). Solution of plant extract was mixed with 95% ethanol, 10% aluminium chloride, 1 M sodium acetate and distilled water. After incubation at room temperature for 30 min absorbance was measured at 415 nm at Shimadzu UV/Vis mini-1240 spectrophotometer. Total flavonoid contents were calculated as quercetin from a calibration curve and expressed as quercetin equivalent (mg/g DM).

Flavanols determination

Flavanols determination was based on the method of Gadzovska et al. (2007) and calibration curve of quercetin. Flavanol contents were expressed as quercetin equivalent (mg/g DM).

Determination of antioxidant activity by the FTC (ferric thiocyanate) method

The inhibitory capacity of extracts was tested against oxidation of linoleic acid by FTC method according to Larrauri et al. (1996). The absorbance was measured spectrophotometrically at 500 nm. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) used as positive control. The percent inhibition of linoleic acid peroxidation was calculated as: (%) inhibition = 100 - [(absorbance increase of sample/absorbance increase of control) × 100].

Antimicrobial bioassay

Antibacterial activity was determined by the disc diffusion method according to the Taylor et al. (1995). Two Gram-positive (*Enterococcus faecalis* ATCC 19433and *Bacillus subtilis* ATCC 6633), two Gram-negative (*Salmonella abony* NCTC 6017 and *Escherichia coli* ATCC 8397) bacterial species and two fungal strains (*Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404) were used. All tested microorganisms were inoculated into respective medium in concentration of 2.0×10^6 colony forming units (cfu/mL). Mueller-Hinton and Sabouraud agar (15 mL), sterilized in a flask and cooled to 45-50 °C, was distributed to sterilized Petri dishes with a diameter of 9 cm. A volume of 10 mL of the sample was injected onto the inoculated discs. The Petri dishes were kept at 4 °C for 2 h, and then incubated at 37 °C

Table 1. Effect of BAP on adventitious shoot and root formation, photosynthetic pigment, total phenolic, total flavonoid, flavanol contents and antioxidative activity of *Mentha piperita*.

Treatment	Number of shoots per explant	Number of roots per explant	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophyll	Carotenoids (mg/g FW)	Total phenolics (mg/g DW)	Total flavonoids (mg/g DW)	Flavanols (mg/g DW)	Antioxidative activity (% of inhibition)
BH	2.467^{b}	3.158^{b}	0.2089^{c}	0.0600^{b}	0.2689^{b,c}	0.0736^{c}	185.63^{b}	70.29^{a}	0.026^{a}	7.69^{d}
B 0.1	3.189^{b}	5.278^{a}	0.3710^{a}	0.1065^{a}	0.4775^{a}	0.1066^{a}	173.63^{c}	75.30^{a}	0.025^{a}	56.64^{a}
B 0.5	4.167^{a}	5.394^{a}	0.2639^{b}	0.0748^{a,b}	0.3387^{b}	0.0875^{b}	51.40^{d}	63.07^{a}	0.018^{b}	36.27^{b}
B 1	2.500^{b}	3.563^{b,c}	0.1631^{c,d}	0.0515^{b}	0.2146^{c,d}	0.0657^{c}	189.13^{b}	24.59^{c}	0.013^{c}	26.67^{c}
B 2	4.167^{a}	3.542^{b}	0.1402^{d}	0.0558^{b}	0.1959^{c,d}	0.0541^{d}	181.27^{b}	69.25^{a}	0.014^{c}	39.15^{b}
B 4	1.400^{b}	1.600^{c}	0.0923^{e}	0.0619^{b}	0.1543^{d}	0.0508^{d}	221.13^{a}	35.35^{b}	0.001^{d}	8.67^{d}
BHA										56.50^{b}
BHT										61.91^{a}

Values are mean (±SD) of three replicates. Treatments not sharing the same letter within one column differ significantly employing parametric (Newman-Keuls) and non-parametric for No of roots and shoots (Kruskal-Walis analysis) tests.
BH-without plant growth regulators; B 0.1-0.1 mg/L BAP; B 0.5-0.5 mg/L BAP; B 1-1 mg/L BAP; B 2-2 mg/L BAP; BHA-butylated hydroxyanisol; BHT-butylated hydroxytoluene.

for 24 h. The diameters of the inhibition zones were measured in mm. Controls were set up with equivalent quantities of methanol. The developing inhibition zones were compared with those of reference discs. Antibiotic chloramphenicol (5 mg/mL) and kanamycin (5 mg/mL) were used as reference.

Statistical analysis

Experimental results were represented as the mean value of the three replicates with standard deviation (SD). The data was analyzed using SPSS 15.0 by employing parametric (Newman-Keuls) and non-parametric (Kruskal-Walis analysis) tests. All statistically significant differences were evaluated at $p<0.05$.

Results and Discussion

Growth parameters and pigment contents

Epicotyls of *M. piperita* inoculated on MS medium supplemented with different concentrations of growth regulators were suitable material for shoot and root induction (Tables 1, 2), with the exception of treatment BI4 (4 mg/L BAP in combination with 0.1 mg/L IBA), which induced necrosis of tissue. Thus, this treatment was not used for further analysis. BAP alone increased the number of shoots comparing to the control. The highest number of shoots (4.167 shoots per explant) was obtained with B0.5 and B2 treatments. However, the shoot number decreased when the highest BAP concentration (4 mg/L) was used for the induction of shoots compared to control, but the changes were not significant (from 1.4 to 2.5). The addition of IBA into the medium considerably enhanced the multiple shoots induction. The maximum induction of multiple shoots (6.490 and 6.241) was achieved from medium BI0.5 and BI2, respectively.

Remarkable improvement was observed when the IBA + BAP combination was used with significant positive correlation between the number of shoots and the plant growth regulator concentration at a level of 5%. Very similar situation was recorded for number of roots. The best rooting response, however, was observed on medium BI0.5, where 8.608 roots were formed per explant. Similarly, the addition of IBA into the medium significantly improved the root induction (treatments BI0.1 and BI0.5). This was expected since the application of auxins enhanced rooting in most species (Benkova and Hejatko 2009; Fukaki and Tasaka 2009). Usually, BAP and kinetin have been reported to be good plant growth regulators for shoot induction from axillary buds and nodal segments when cultured on MS medium (Rech and Pires 1986; Sunandakumari et al. 2004). However, in some plant species, including *M. spicata*, *M. arvensis,* and *Lavandula viridis*, a combination of cytokinin and auxin in the medium was more effective (Hirata et al. 1990; Kukreja et al. 1991; Dias et al. 2002).

Some hormonal treatments stimulated significant increases in the chlorophyll a and total chlorophyll contents (Tables 1, 2). The highest chlorophyll a content (0.3710) was obtained in treatment B0.1 followed by BI2 (0.2989), B0.5 (0.2639) and BI0.5 (0.2556). Similar situation was found in total chlorophyll content. Higher concentrations of BAP alone decreased chlorophyll b and carotenoids content (Table 1), while the addition of IBA induced the opposite effect (Table 2), where the highest concentration of BAP (2 mg/L) in combination with IBA increased the level of photosynthetic pigments (BI2 treatment). It was not possible to find a direct link between concentrations of plant growth regulators used

Table 2. Effect of BAP and IBA on adventitious shoot and root formation, photosynthetic pigment, total phenolic, total flavonoid, flavanol contents and antioxidative activity of *Mentha piperita*.

Treatment	Number of shoots per explant	Number of roots per explant	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophylls	Carotenoids (mg/g FW)	Total phenolics (mg/g DW)	Total flavonoids (mg/g DW)	Flavanols (mg/g DW)	Antioxidative activity (% of inhibition)
BH	2.467^{b}	3.158^{c}	0.2089^{c}	0.0600^{b}	0.2689$^{b.c}$	0.0736^{d}	185.63^{a}	70.29$^{b.c}$	0.026^{c}	7.69^{d}
BI 0.1	3.967$^{a.b}$	7.400$^{a.b}$	0.2175$^{b.c}$	0.0700$^{a.b}$	0.2875$^{b.c}$	0.0731^{d}	168.99^{b}	121.42^{a}	0.046^{a}	23.14^{c}
BI 0.5	6.490^{a}	8.608^{a}	0.2556$^{a.b}$	0.0834$^{a.b}$	0.3390^{b}	0.0868^{b}	61.19^{d}	57.00^{c}	0.019^{d}	24.20^{c}
BI 1	4.211$^{a.b}$	6.278$^{a.b.c}$	0.1692^{d}	0.0569^{b}	0.2261^{c}	0.0619^{c}	143.40^{c}	58.63^{c}	0.033^{b}	63.33^{a}
BI 2	6.241^{a}	4.481$^{b.c}$	0.2989^{a}	0.1029^{a}	0.4018^{a}	0.0979^{a}	167.60^{b}	77.06^{b}	0.033^{b}	50.82^{b}
BHA										56.50^{b}
BHT										61.91^{a}

Values are mean of three replicates. Treatments not sharing the same letter within one column differ significantly employing parametric (Newman-Keuls) and non-parametric for No of roots and shoots (Kruskal-Walis analysis) tests.
BH-without plant growth regulators; BI 0.1-0.1 mg/L BAP + 0.1 mg/L IBA; BI0.5-0.5 mg/L BAP + 0.1 mg/L IBA; BI1-1 mg/L BAP + 0.1 mg/L IBA; BI2-2 mg/L BAP + 0.1 mg/L IBA; BHA-butylated hydroxyanisol; BHT-butylated hydroxytoluene.

and the content of photosynthetic pigments (Edelman and Hanson 1971; Karalija and Parić 2011; Karalija et al. 2016). Cytokinins as well as auxins can improve concentration of photosynthetic pigments (Verma and Sen 2008; Parsaeimehr et al. 2010; Vamil et al. 2010). Changes in the chlorophyll content, caused by plant growth regulators could be related to growth rate, primary and secondary metabolic activities (Lichtenthaler 1987) as shown in treatments B0.5, BI0.5 and BI2.

Phenolic content and antioxidative properties

Application of BAP alone in highest concentration significantly increased production of total phenolics and all other treatments induced similar or decreased total phenolics contents. Addition of IBA in the medium decreased total phenolics content.

Among all applied treatments only BI0.1 increased flavonoid contents. Flavanols content also varied depending of treatment. A 2-fold increase on BI0.1 treatment was observed (Tables 1,2).

The effects of plant growth regulators on secondary metabolite production in *in vitro* culture systems are highly variable (Khan et al. 2008; Santoro et al. 2013), depending of the plant species. For example, BAP and IBA stimulated the production of total phenolics and flavonoids in *Thymus vulgaris* and *Origanum vulgare*, but decreased it in *Ocimum basilicum* (Karalija and Parić 2011; Karalija et al. 2016). On the other hand, BAP alone increased the total yield of essential oils and its components (menthone, menthol, pulegone, and menthofuran) in *M. piperita*, while the combination of BAP and IBA did not significantly change the production of plant secondary compounds (Santoro et al. 2013).

A single plant hormone may regulate a wide range of physiological and growth processes, or a particular process may be regulated by the action of many plant hormones (Santoro et al. 2013). Chemical changes were evident in some cases, but not in others. BAP applied alone increased total phenolics content only in highest concentration (Tables 1, 2). All combinations of BAP and IBA decreased the production of phenolic compounds when compared to control.

In the present study, the antioxidant activity of *M. piperita* extracts were determined by peroxidation of linoleic acid using the ferric thiocyanate method (FTC) and significant differences between various treatments were determined (Tables 1, 2). It was found that all the treatments were significantly different comparing to control (BH). A 6-fold increase of antioxidant activity was noticed in treatment BI1 (63.33), which was more effective than two positive probes, followed by B0.1 (56.64) and BI2 (50.82). These results indicate that these extracts can significantly inhibit the peroxidation of linoleic acid and reduce the formation of hydroperoxide, thus implying that antioxidative activity of *M. piperita* could be successfully improved by hormone type/ratio manipulation. Antioxidant activities from aromatic plants are mainly associated with the active compounds present in their tissues. But, since it is a complex mixture of various molecules this can be due to the high percentage of main constituents, but also to the synergistic and antagonistic effect between these main constituents and other constituents that can be present in small quantities (Ćavar et al. 2012). To the best of our knowledge, this is the first report of effect of plant growth regulators on antioxidative activities of *M. piperita* using FTC method, but antioxidant activity of *M. piperita* essential oils, using other methods of determination has previously been reported (Mimica-Dukic et al. 2003; Derwich et al. 2011).

Antimicrobial activity

Results obtained in the present study revealed that the dif-

Table 3. Effect of BAP alone or in combination with IBA on antimicrobial activity of different *M. piperita* extracts.

Treatment	*E. coli*	*S. abony*	*E. faecalis*	*B. spizizenii*	*A. brasiliensis*	*C. albicans*
BH	-	-	-	-	-	-
B 0.1	-	-	-	-	11.3	11.7
B 0.5	14.0	13.0	-	-	14.0	11.0
B 1	-	-	-	-	12.7	11.0
B 2	-	-	-	-	12.7	9.5
B 4	-	-	-	-	12.0	9.7
BI 0.1	10.3	-	-	-	13.3	10.0
BI 0.5	9.3	-	-	-	12.0	-
BI 1	9.0	-	-	-	14.3	10.7
BI 2	-	-	-	-	14.7	10.5
Standards						
Amphothericin B	20.0					
Chloramphenicol	17.7					
Nystatin	17.0					

Values are mean inhibition zone (mm) ± SD of three replicates. (-) No zone of inhibition.
BH-without plant growth regulators; B 0.1-0.1 mg/L BAP; B 0.5-0.5 mg/L BAP; B 1-1 mg/L BAP; B 2-2 mg/L BAP; BI 0.1-0.1 mg/L BAP + 0.1 mg/L IBA; BI0.5-0.5 mg/L BAP + 0.1 mg/L IBA; BI1-1 mg/L BAP + 0.1 mg/L IBA; BI2-2 mg/L BAP + 0.1 mg/L IBA.

ferent tested plants extracts possess potential antibacterial activity against *E. coli* and antifungal activity against *A. brasiliensis* and *C. albicans* (Table 3). The antimicrobial activities of the all extracts showed no inhibition activity against Gram-positive strains tested (*E. faecalis*, *B. spizizenii*). Both fungi, *A. brasiliensis* and *C. albicans* appeared to be sensitive to the all tested extracts and only the Gram-negative *E. coli* was sensitive to some of them (B0.5, BI0.1, BI0.5, and BI1). The extracts of B0.5 treatments were active against Gram-negative *S. abony*. *A. brasiliensis* and *C. albicans* appeared to be the most sensitive strains followed by Gram-negative *E. coli*. Our results indicate that plant growth regulators can enhance antimicrobial activity since control plants, showed no activity against tested microbial strains. Similar results were obtained, by other authors, who reported antimicrobial activity of different *Mentha* species (oils) (Abdel Moneim et al. 2011; Basheer and Abdullah 2013). Numerous studies have confirmed that plant growth regulators in medium can affect the antibacterial properties of plants (Gibbons 2004; Pitta-Alvarez et al. 2008; Karalija et al. 2016).

Conclusions

IBA and BAP alter secondary metabolite production in sterile cultures by changing both primary and secondary metabolism of plants. This study showed that application of plant growth regulators is a good option for stimulating secondary metabolites production, but selection of the right plant hormone and its optimal concentrations are crucial for increasing secondary metabolite production and bioactivity of plant extracts.

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