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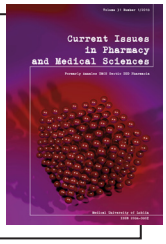

In vitro propagated *Mentha rotundifolia* (L.) Huds and antioxidant activity of its essential oil

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ABSTRACT

Mentha rotundifolia (L.) Huds is an aromatic plant used for its medicinal values. This study aims to select appropriate conditions for *in vitro* propagation of *M. rotundifolia* (L.) Huds and to evaluate yield and antioxidants activity of its essential oils (EOs).

The explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of growth regulators 6-benzylaminopurine (BAP) and Gibberellin (GA_3). Hydrodistillated EOs obtained from acclimatized and mother plant, were evaluated for their antioxidant activity. Tests were performed on DPPH free radical-scavenging, ABTS and CUPRAC assays.

Shoot induction and multiplication were successfully carried out on MS medium supplemented with the following hormones combinations: 1 mg/l BAP, 1 mg/l GA_3 and 0.5 mg/l BAP, 0.5 mg/l GA_3, respectively. Stem length, nodes and leaves number measured from development *vitroplant* were 6.89 cm, 5.22 nodes and 11.92 leaves per *vitroplant*, respectively.

In vitro rooted plants were successfully acclimatized at a temperature of 23 ± 2°C and a long day photoperiod with a total survival rate exceeding 95%.

EO yield of acclimated plant varied between (0.88-1.49 ml/100 g dry matter) compared to wild plant (0.73 ml/100 g dry matter). The antioxidant potential of EOs from acclimated plant showed on DPPH free radical-scavenging, ABTS and CUPRAC assays values of(IC_{50}:4.18-24.93mg/ml),(IC_{50}:0.51-1.56mg/ml)and($A_{0.50}$:0.34-2.71mg/ml),respectively. In contrast, the wild plant exhibited on the same tests the values of (IC_{50}: 10.35 mg/ml), (IC_{50}: 0.12 mg/ml) and ($A_{0.50}$: 0.99 mg/ml), respectively.

The results suggest that micropropagation of *M. rotundifolia* (L.) Huds can be an interesting alternative for producing important plant material with the possibility to modulate EO yield and its antioxidant potential for future commercial purposes.

INTRODUCTION

Essential oils are of interest as potential sources of bioactive natural molecules. Many spices, herbs and extracts have antioxidant and antimicrobial activity that is almost invariably due to the essential oil fraction. This is often a complex mixture of different compounds, some of which possess these properties [1].

Faced with the limits of conventional sources (picking and cultivation plants) for the production of secondary metabolites and the limits of those for the chemical synthesis of bioactive molecules, some industrial sectors have called on scientists to take into account the potential of biotechnology and *in vitro* cell cultures as an alternative source of supply for the production of high value-added substances. This option stems from the ability of plant cells to be totipotent, i.e. the property of having all the information of the mother plant, especially its metabolic capacity. [2]. Indeed, *in vitro* culture offers the advantage of controlling environmental conditions and thus modulating production according to demand [3,4].In most of the cultures studied,

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the slow growth rate of plant cells and the low levels of biomolecules produced are obstacles to overcome in order for plant cell culture to be economically viable [5]. On these aspects, various strategies were followed to optimize the production of biomass and the levels of secondary metabolites produced by *in vitro* cell cultures, namely, the selection of highly productive lines, the optimization of growing conditions and the induction of metabolic pathways [6].

This work deals with *in vitro* propagation of *Mentha rotundifolia* (L.) Huds and is aimed to study the effect of acclimatization conditions (temperature and photoperiod) on the yield of essential oil and on its antioxidant activity.

MATERIAL AND METHODS

Chemicals

Chemicals of MS culture medium, Anhydrous sodium sulfate (Na_2SO_4), 6-benzylaminopurine (BAP), Gibberellin (GA_3), Polyoxyethylenesorbitan monolaurate (tween 20), 2,2-diphenyl-β-picrylhydrazyl (DPPH), Butylated hydroxyanisole (BHA), 2,2'-azynobis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), potassium persulfate, methanol, copper (II) chloride, neocuproine alcoholic and ammonium acetate were purchased from Sigma-Aldrich (Steinheim, Germany).

Plant Material

Mentha rotundifolia (L.) Huds plants were collected at the flowering stage in June 2014, at Beni Hmiden, Constantine. The species identity was confirmed by Hocine Laouer, Professor at the Department of Biology and Plant Ecology, Ferhat Abbas University of Setif. A voucher specimen was deposited under N° MR0830 in the herbarium of C.R.Bt, Constantine (Algeria). Plant materials were dried at room temperature and stored until extraction and analysis.

Establishment of plant tissue culture

Explants of wild *Mentha rotundifolia* (L.) Huds were washed thoroughly with tap water. They were then disinfected with sodium hypochlorite 10% solution containing 2 drops of tween 20 (Polyoxyethylenesorbitan monolaurate) for 10 min before dipping in 70% ethanol for 30 sec. Sterilized material was then rinsed with sterile distilled water.

After disinfection, nodal fragments were cultured on MS [7] agar medium (0.8%) supplemented with sucrose (3%) and different hormonal combinations (6-benzylaminopurine BAP: 0, 0.5, 1 mg/l and Gibberellin GA3:0, 0.5, 1 mg/l). Culture media were sterilized by autoclaving at 121°C for 20 min. Culture flasks were maintained in a growth chamber at 23°C with a photoperiod 16h/8h (light/dark). Established cultures were maintained with renewal of medium each 4 weeks.

Shoot multiplication

Shoot culture multiplication was carried out with MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l GA_3. Subcultures were performed at a frequency of 3-4 weeks. The stem length, numbers of nodes and leaves per *vitroplant* were determined after 30 days of culture.

Condition of acclimatization

Vitroplants with well-developed roots were carefully recovered, washed with distilled water and transferred into pots (7 cm in diameter) containing a sterilized mixture of sand and peat (1:1, v/v). The pots were placed in an acclimatization chamber at a temperature of 23±2°C with a photoperiod of 16h/8h (light/dark) and a humidity of 70 %. The plantlets were then sprayed with distilled water 3 to 4 times in day during the first week and then irrigated twice a week. The irrigation was carried out in an alternating manner between distilled water and an irrigation mineral solution.

After 70 days of acclimatization (estimated flowering period under conditions of 23±2°C, photoperiod of 16h light and 70% humidity), the plant lets were exposed to abiotic treatment (temperature, photoperiod) for one week (7 days) to evaluate its influence on the yield and antioxidant activity of EOs.

The studied parameters were:

- temperature: 35±2°C and 16±2°C *vs.* 23±2°C (control),
- photoperiod: 8h/16h (light/dark), continuous dark *vs.* 16h/8h (light/dark) (control).

Extraction of essential oils

After acclimatization, EOs from wild and acclimated plants were obtained by hydrodistillation using a Clevenger type apparatus. Dry plant material (100 g) was introduced into a 2l flask filled with 1l of distilled water and distilled for 3 hours. EOs were treated with anhydrous sodium sulfate (Na_2SO_4) and stored in sealed glass vials at 4°C.

Antioxidant activity

Three methods were used to evaluate the antioxidant activity: DPPH free radical-scavenging, ABTS and CUPRAC, under conditions described below.

Free radical scavenging activity test

The free radical scavenging activity of EOs was measured using 2,2-diphenyl-β-picrylhydrazyl (DPPH) [8], while Butylated hydroxyanisole (BHA) was employed as positive control. A two-fold serial dilution from stock extract solution (4 µg/ml) was carried out with methanol to obtain test solutions in the range of 10 to 0.156 mg/ml. 160 µL of DPPH methanol solution (4×10^{-5}M) was added to 40 µL of sample solutions of different concentrations and butylated hydroxyanisole solution separately. All tests were carried out in triplicate (n = 3). The reaction mixture was shaken and then incubated for 60 min at room temperature. The amount of DPPH remaining was determined at 517 nm against a blank (methanol with DPPH) using a 96-well micro plate reader, spectra (Perkin Elmer, EnSpire Multilabel Reader 23001154).

Absorbance was recorded and % **reduction** was calculated using the formula given below:

$$\%\ \text{reduction of DPPH} = \frac{A_{cont} - A_{samp}}{A_{cont}} \times 100,$$

where:

A_{cont} – absorbance of the blank
A_{samp} – absorbance of sample.

IC_{50} was determined from the equation obtained by plotting the linear curve of reduction percentages against concentrations. DDPH free scavenging ability was considered significant if the IC_{50}-value was lower [9].

ABTS cation radical decolorization assay

The ABTS scavenging activity was done by the method of Re *et al*. [10] with slight modifications. The ABTS (2, 2'-azynobis-[3-ethylbenzothiazoline-6-sulfonic acid]) was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, then stored in the dark at room temperature for 12h. The oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6h had elapsed. The radical cation was stable in this form for more than 2 days with storage in the dark at room temperature. Before usage, the ABTS solution was diluted to get an absorbance of 0.700±0.020 at 734 nm with ethanol. Subsequently, 160 µL of ABTS solution were added to 40 µL of sample solution in methanol at different concentrations (10 mg/ml to 0.156 mg/ml). After 10 min, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance (methanol). The scavenging capability of ABTS was calculated using the following equation:

$$\text{ABTS scavenging activity (\%)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100.$$

Cupric reducing antioxidant capacity (CUPRAC) assay

CUPRAC was determined according to the method developed by Apak *et al*. [11]. The method comprises mixing of 40 µL of antioxidant solution with 50 µL of a copper (II) chloride solution, 50 µL of neocuproine alcoholic solution, and 60 µL of ammonium acetate aqueous buffer at pH 7. After 60 min, the absorbance was read at 450 nm.

Statistical analysis

All the measurements were replicated three times for each assay and the results are presented as mean ± SD. Statistical comparisons were done using the SPSS program and the analyses of variance (ANOVA) test. P-values ($P < 0.05$) were regarded as statistically significant.

RESULTS

Vitroplants establishment

The effect of the different hormonal balances on shoot induction is shown in Table 1.

Table 1. Effect of BAP and GA_3 on shoot induction of *Mentha rotundifolia* (L.) Huds

Medium	Hormonal Balances	Vitroplants characteristics
M1	0 mg/l BAP,0 mg/l GA3	Short shoots with serried nodes
M2	0.5 mg/l BAP,0 mg/l GA3	Medium-sized shoots with serried nodes
M3	0.5 mg/l BAP,1 mg/l GA3	Medium-sized shoots with well separated nodes
M4	1 mg/l BAP,0 mg/l GA3	Long shoots with serried nodes
M5	1 mg/l BAP,1 mg/l GA3	Long shoots with well separated nodes

As revealed, induction of shoots was successfully carried out on MS medium supplemented with 1 mg/l BAP, 1 mg/l GA_3.

Culture multiplication

The most effective shoot multiplication was achieved with MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l GA_3. An average stem length (6.89±3.62 cm); number of nodes (5.22±1.77) and leaves (11.92±8.13) were obtained.

Acclimatization

The acclimatization protocol used for *in vitro* rooted plants was successfully established with a total survival rate of 96-100 %.

Essential oils yields

Essential oils contents from acclimated and wild plant are shown in Table 2.

Table 2. Yield of essential oils from wild plant and acclimated plants

Plant material	Culture conditions (Temperature, light/dark)	Yield (ml/100 g dry matter)
Wild plant	/	0.73±0.06 bc
Culture A	(23°C, 16h/8h)	0.89±0.09 b
Culture B	(16°C, 16h/8h)	0.88±0.07 b
Culture C	(35°C, 16h/8h)	**1.48±0.01** a
Culture D	(23°C, 8h/16h)	**1.49±0.25** a
Culture E	(23°C, total darkness)	0.97±0.01 b

Note: All the measurements were replicated three times for each assay and the results are presented as mean ± SD Superscript letters indicate significant differences according to Tukey's test (P≤0.05)

As shown in Table 2, the EOs contents were significantly raised by increasing the temperature (from 23°C to 35°C) in culture C (1.48%) or shorting the light exposure (from 16h to 8h) in culture D (1.49%), compared to control (Culture A, 0.89%). Culture E, maintaining at total darkness, led to no significant change in the yield (0.97%).

Antioxidant activity

The result of antioxidant activity, expressed by IC_{50} (DPPH free radical-scavenging, ABTS) and $A_{0.50}$ (CUPRAC) are shown in Table 3.

Table 3. Antioxidant activity of essential oils measured by DPPH, ABTS and CUPRAC assays

Essential oils (conditions)	DPPH IC_{50} (mg/ml)	ABTS IC_{50} (mg/ml)	CUPRAC $A_{0.50}$ (mg/ml)
Wild plant	10.35±0.58	0.12±0.09	0.99±0.03
Culture A (23°C, 16h/8h)	4.75±0.31	0.51±0.02	1.80±0.34
Culture B (16°C, 16h/8h)	4.18±0.29	1.53±0.18	1.81±0.14
Culture C (35°C, 16h/8h)	24.93±8.80	0.92±0.43	1.21±0.06
Culture D (23°C, 8h/16h)	9.19±0.18	1.56±0.14	2.71±0.08
Culture E (23°C, total darkness)	7.44±0.19	0.85±0.12	0.34±0.15
BHA[b]	0.006±0.10	0.005±0.77	0.008±0.36

[a] Values are expressed as mean ± standard deviation
[b] Reference components

As revealed in Table 3, DPPH scavenging effect of BHA was higher than that in wild and acclimated plants. Moreover, the results obtained reveal that essential oil of wild plant *M. rotundifolia* (L.) Huds. has a medium antioxidant potential (IC_{50}: 10.35±0.58 mg/ml).

The study of the effect of the acclimatization conditions (Temperature and photoperiod) on the antioxidant activity showed an increase in the antioxidant potential of essential oils (IC_{50}: 4.18±0.29 mg/ml) and (IC_{50}: 4.75±0.31 mg/ml) for plantlets grown at 16°C (Culture B) and 23°C (Culture A), respectively. Low antioxidant potential was recorded for plantlets grown at 35°C (Culture C) (IC_{50}: 24.93±8.80 mg/ml).

Photoperiod also has a positive effect on the antioxidant activity of the essential oils of acclimated *M. rotundifolia* (L.) Huds (IC_{50}: 9.19±0.18 mg/ml) and (IC_{50}: 7.44±0.19 mg/ml) for plantlets grown at 16h/8h (dark/ light) (Culture D) and total darkness (Culture E), respectively.

In the ABTS assay (Table 3), BHA was the most effective antioxidant agent (IC50: 0.005±0.77 mg/ml) than the other tested plant materials. All EOs have a moderate-scavenging activity. Herein, essential oil of wild plant *M. rotundifolia* (L.) Huds showed the most important antiradical activity (IC_{50}: 0.12±0.09 mg/ml) followed by EO from plantlets grown at 23°C (Culture A), EO from plantlets grown at 35°C (Culture C) and EO from plantlets grown at total darkness (Culture E) (IC_{50}: 0.51±0.02, 0.92±0.43 and 0.85±0.12 mg/ml, respectively). EO from plantlets grown at 16°C (Culture B) and EO from plantlets grown with photoperiod of 16 h /8 h (darkn/lights) (Culture D) also showed a moderate activity.

The CUPRAC assay shows similar results than ABTS test. All EOs have a moderate-scavenging activity, but less effective than the synthetic antioxidant (BHA) ($A_{0.50}$: 0.008±0.36 mg/ml).

Wild plant M. *rotundifolia* (L.) Huds essential oil ($A_{0.50}$: 0.99±0.03 mg/ml) exhibited a higher activity, followed by EO from plantlets grown at total darkness (Culture E) ($A_{0.50}$: 0.34 ± 0.15 mg/ml). EO from plantlets grown at 35°C (Culture C) ($A_{0.50}$: 1.21 ± 0.06 mg/ml), EO from plantlets grown at 23°C (Culture A) ($A_{0.50}$: 1.80 ± 0.34 mg/ml), EO from plantlets grown at 16°C (Culture B) ($A_{0.50}$: 1.81±0.14 mg/ml) and EO from plantlets grown with photoperiod of 16 h /8 h (darkn/ lights) (Culture D) ($A_{0.50}$: 2.71±0.08 mg/ml) were also found to be active.

DISCUSSION

The micropropagation technique can be an interesting alternative to wild grown plants for producing important plant material and phytochemicals (12). The success of the technique depends among others on the balance between different phytohormones [12]. The most important factor influencing bud differentiation is the type of growth regulator used especially the type of cytokinin [2,13]. Its concentration in the culture medium is critical for shoot organogenesis [14]. The promoter effect of BAP in the induction of multiple shoots has previously been reported [15]. Ours results showed that MS cultured medium supplemented with various concentrations of cytokinin led to different responses of nodal explants. The increase in BAP concentration in culture media induced the formation of more shoots from the axillary buds. Thus, bud burst and shoot proliferation in nodal explant cultures is a function of cytokinin activity. As found in our testing, the best result was obtained by culturing the explants on MS medium supplemented with BAP at a concentration of 1 mg/l. The axillary buds even start in an MS medium free of BAP, which can be attributed to the high concentration of endogenous cytokinin in mint explants [13].

As far as shoot multiplication is concerned, cytokinin requirements were low compared to those required for culture initiation. Among the various concentrations of BAP tested, 0.5 mg/l was the most appropriate for shoot multiplication of *M. rotundifolia* (L.) Huds. Decreasing BAP concentrations from a high dose (1 mg/l) for the establishment phase to a reduced dose (0.5 mg/l) for the multiplication phase improves stem proliferation and quality. This may be due to the cytokinin residues present in the stems at the multiplication stage. The use of low doses of cytokinin during the multiplication step has also been reported to be essential for promoting proliferation and multiplication in many other plants [16]. Echeverrigaray *et al*. [16] reported a high multiplication rate of *Lavandula dentata* using MS medium supplemented with 2.2 µM (equivalent of 0.5 mg/l) BA with an average shoot length of 3.53 cm.

The distance between the nodes is essential because they serve as base of fixation for the next subcultures [13]. The results obtained showed that GA_3 was effective on stem elongation and an inducing effect of elongation was observed after 3-4 weeks of culture and the best result was MS medium supplemented with 1 mg/l GA_3. The promoter effect of GA_3 on the elongation of shoots generated on medium containing BAP has been reported in several species [15,17,18].

Mechanisms that interfere with root formation are still poorly understood [13]. However, the success of this step is reported to be related to several factors [13]. Since the shoots rooted spontaneously, no other treatment with auxins was needed to promote rooting. This is reported previously by Zezurate *et al*. [19].

The acclimatized plants appeared uniform and showed no detectable variation as previously in other studies [15,20].

EO content of *M. rotundifolia* (L.) Huds in wild plants was 0.73±0.06% (v/w). This yield obtained at blooming stage is comparable within the values (0.7-0.9%) reported by other authors from Algerian wild *M. rotundifolia* species [21]. The results obtained show that the plants which underwent thermal or luminous treatments gave significantly higher yields of essential oils compared to those of wild plant material and control (culture A). Hirata *et al*. [22] reported similar yield of EO from *vitroplants* (0.9-1.3 mg/g) and mother plant of *M. spicata* (1-1.5 mg/g). However, other studies have reported that the EO yield *in vitro* was higher than that obtained from wild plants. Arafeh *et al*. [23] reported that the quantity of EO extracted from *in vitro Origanum vulgare* was six times greater than that of the wild plant.

The antioxidant activity of essential oils could be attributed to their chemical composition [24] which is determined by genotype, and influenced by environmental and

agronomic conditions [25]. The relationship between antioxidant activity and their chemical profiles has already been reported [26].

The essential oils tested showed antioxidant activity but less as compared to the synthetic antioxidant (BHA). Economically, it is desirable to conduct a thorough analysis of the mechanisms of action of these compounds and a more advanced research on the synergy of basic compounds and the association of extracts of essential oils in food products. In the end, these natural properties of essential oils make them very promising preservatives for the food industry.

CONCLUSIONS

In vitro culture is, therefore, an interesting approach that has many advantages over conventional methods of selection. In addition, metabolic engineering opens up promising new prospects for improving the production of secondary metabolites, enabling the plant medicinal market to bring a biological preservative (essential oil) for the food industry which comes from the local biological heritage.

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