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ORIGINAL PAPER

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In vitro MULTIPLICATION OF *Mentha piperita* L. AND COMPARATIVE EVALUATION OF SOME BIOCHEMICAL COMPOUNDS IN PLANTS REGENERATED BY MICROPROPAGATION AND CONVENTIONAL METHOD

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ABSTRACT

The aim of this study was to elaborate an efficient *in vitro* multiplication protocol for *Mentha piperita* L. (peppermint) and to perform a comparative evaluation of some biochemical compounds in plants regenerated by micropropagation and conventional method. The use of a plain Murashige and Skoog (MS) basal medium favored the induction of regenerative processes, the percentage of explants that started to grow four weeks after inoculation being 92%. The highest multiplication rate (7.12 shoots/explant) and the highest average shoot length (8.11 cm) were obtained on the MS medium supplemented with 1 mg/L benzylaminopurine, when nodal fragments were used as explants. The rooting phase was not necessary, the shoots developing roots on the multiplication medium. The acclimatization rate of *in vitro* regenerated plants to *ex vitro* conditions was 96%. Although biochemical investigations revealed some differences between *in vitro* regenerated plants and those obtained by conventional methods, the results obtained show that micropropagation can be used successfully to obtain high-quality peppermint biological material, a potential source of bioactive compounds with therapeutic effect.

Key words: peppermint, tissue cultures, growth regulators, photosynthetic pigments, soluble carbohydrates, total polyphenols

INTRODUCTION

Mentha piperita L. (peppermint) belonging to the family *Lamiaceae* is a perennial plant with important therapeutic effects: antioxidant, antiviral, antibacterial, spasmolytic, carminative, antiseptic, antiparasitic, analgesic, and antitumor [Trevisan et al. 2017].

Given its therapeutic potential, there is currently an increase in global demand for peppermint biological material. The ruthless exploitation has led to a severe reduction in the natural resources of this valuable medicinal plant. Advances in biotechnological approaches have provided a set of techniques that contribute to solving the problems of extinction or genetic erosion of plants. Micropropagation has proven to be an excellent method for the mass production of plants of horticultural, economic, and medicinal importance [Pati et al. 2006]. Compared to traditional multiplication methods, the advantages of micropropagation are that a large number of healthy plantlets can be obtained in

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a short time, in small physical spaces, at any time of the year, regardless of the latency phenomena [Santoro et al. 2013].

Regeneration of plants from different types of explants, either by direct organogenesis or by callogenesis, on different strength of Murashige and Skoog (MS) medium [Murashige and Skoog 1962], with or without the use of plant growth regulators has been previously reported in *Mentha piperita* [Ghanti et al. 2004, Sarwar et al. 2009], *Mentha spicata* [Fadel et al. 2010, Ozdemir 2017] and *Mentha viridis* [Raja and Arockiasamy 2009, Senthil and Kamraj 2012]. However, some of the serious limitations of the protocols mentioned above were the low frequency of regeneration, the small number of shoots and roots, the appearance of the callus phase during organogenesis, and the low survival rate of *in vitro* regenerated plants after *ex vitro* transfer.

Therefore, the aim of this study was to elaborate an efficient method of peppermint micropropagation that could ensure a high frequency of regeneration in a short time and a high survival rate of plants in the acclimatization phase. We also evaluated the content of some biochemical compounds in plants regenerated *in vitro* and compared it with that in plants obtained by conventional method.

MATERIALS AND METHODS

Initiation of *in vitro* culture. The explants used for the initiation of *in vitro* cultures consisted of shoot tips and nodal fragments from actively growing shoots of *Mentha piperita* L. mother stock plants. The shoots were first rinsed in tap water and sterilized in 6% calcium hypochlorite solution for 10 min, followed by 3 rinses using sterile distilled water. The stem segments were then cut with a sterile scalpel blade into smaller segments (1–1.5 cm long), each with one node used as explants. The explants were placed vertically on a plain MS medium [Murashige and Skoog 1962] and maintained as shoot tip and single node cultures until the plant material was sufficient for further experiments. The inoculation of the explants was carried out under aseptic conditions using a laminar air flow hood.

At this stage of the experiment, as well as in subsequent stages, the culture medium was supplemented with 40 g/L glucose, 32 mg/L NaFeEDTA, and 7 g/L agar. The culture media were sterilized by autoclaving at 120°C for 20 min. Prior to autoclaving, the pH of the medium was adjusted to 5.6–5.8 with 1N KOH or 1N HCl. All cultures were transferred in a growth room with controlled conditions at 22–24°C, a light photoperiod of 16 h at 3000 lx. In the culture initiation phase, the evaluated parameter was the percentage of explants that started to grow, one month after inoculation.

***In vitro* multiplication stage.** Proliferation of shoots was induced on a full-strength MS medium supplemented with different types of cytokinins (benzylaminopurine – BAP, 2 isopentyladenine – 2iP, kinetin – KIN) at various concentrations (1, 2, and 5 mg/L). Subculturing was performed every four weeks. The number of shoots per explant and the length of the shoots were monitored as growth parameters.

The experimental design was planned in triplicates for each treatment. Statistical interpretation of the data was done using SPSS 10 for Windows program. The differences between the variants compared to the control were analyzed with One Way ANOVA – LSD, considering to be significant at $P < 0.05$.

The rooting phase was not necessary, the shoots developing roots on the multiplication medium.

Acclimatization stage. The *in vitro* rooted plantlets were removed from the culture medium and their roots were washed in running tap water and then transplanted in pills of peat (Jiffy) for acclimatization to *ex vitro* conditions. To ensure a high humidity environment, the plants were placed under a tunnel of plastic foil and spraying them with water until they start to harden. The percentage of acclimatized plants (the ratio between the number of viable plants and the total number of plants transferred *ex vitro*) was calculated after four weeks. The acclimatized plants were then transplanted in pots for fortification and maintained in a greenhouse for further growth and development.

Multiplication of peppermint by conventional methods. In order to obtain peppermint biological material by conventional methods, seeds of *Mentha piperita* L. were sown in peat. One month after the start of the experiment, the seed germination rate was calculated as a percentage of germinated seeds.

Biochemical analysis. Biochemical determinations were performed on peppermint plants regenerated

by micropropagation (V1) and conventional method (V2). To determine the *free water content*, the plant material was dried at 40°C to constant weight. To determine the *total water content*, the plant material was dried at 105°C to constant weight. The *bound water content* of the analyzed samples was calculated by difference, according to the relation: bound water (%) = total water (%) – free water (%). The *dry matter content* was expressed as a percentage and calculated by the formula: dry matter (%) = 100 – total water (%).

The *photosynthetic pigments* were extracted with 85% acetone [Holm 1954]. 0.1–0.2 g green material was cut into small pieces (2–3 mm), milled and mixed with 2–3 mL of 85% acetone. The obtained extract was filtered, and the volume of the extract was then brought to 25 mL, with 85% acetone. Absorbance measurements were read using a spectrophotometer (Thermo Scientific Biomate 5) at 440.5 nm, 644 nm, and 662 nm wavelengths. The content of chlorophyll *a*, chlorophyll *b*, and carotenoid pigments was calculated with the formulas proposed by Holm [1954] and expressed in milligram/gram (mg/g) green substance.

The dosage of soluble carbohydrates and total polyphenols was performed in extracts obtained using a MAS-II microwave synthesis and extraction system (Hanon Instruments, Shanghai, China). The fresh plant material was triturated by gradually adding the extraction solvent (ethanol 70%, v/v). The plant material : solvent ratio was 1 : 10 (m/v). The microwave extraction was performed at 40°C for 10 min. The microwave power was controlled and maintained at 250W and magnetic stirring at 200 rpm. The plant extracts were then filtered.

The dosage of *soluble carbohydrates* from the plant material was performed by the colorimetric method, with anthrone reagent [Pánczél and Eifert 1960]. 0.5 mL of the extract was mixed with 0.5 mL of distilled water and 2 mL of anthrone reagent. The obtained mixture was boiled for 10 min, after which it was cooled for 15 min. Absorbance was read at 620 nm using a spectrophotometer. The soluble carbohydrates content was expressed as mg/g green substance.

The *total phenolic content* was spectrophotometrically evaluated using Folin-Ciocalteu reagent [Singleton and Rossi 1965]. 1 mL of alcoholic extract, 75 mL of distilled water, and 5 mL of Folin-Ciocalteu reagent were placed in a 100 mL flask. After 3 min, 10 mL of 20% sodium carbonate was added and filled with distilled water to the mark. The mixture was allowed to stand for 60 min at room temperature, in the dark. Thereafter, the absorbance was measured at 765 nm. Tannic acid was used as standard and the results were expressed as tannic acid equivalents/fresh weight (mg TAE/g sample).

Each biochemical analysis was performed in three repetitions.

RESULTS

Morphogenetic reaction of peppermint explants in *in vitro* culture. The inclusion of peppermint in the *in vitro* culture system does not pose particular problems, the use of calcium hypochlorite (6% solution for 10 min) for sterilization of biological material proved to be efficient. The plain MS basal medium favored the induction of regenerative processes, four weeks after the start of the experiment there was a percentage of 92% explants that started to grow.

Regeneration of shoots was influenced by both the type and concentration of cytokinin and the type of explant used. In general, the regenerative potential of nodal fragments was superior to the regenerative potential of shoot tip explants.

Statistical interpretation using the SPSS 10 program of our results showed that, for both types of explants used, supplementation of the nutrient medium with 1 mg/L BAP induced an increase compared to the control of both the number of shoots/explant and the length of the shoots, in the case of the shoot tip explants the increases being significant. The increase in BAP concentration led to a weaker morphogenetic response. The highest values of the multiplication rate (7.12 shoots/explant) and of the length of the shoots (8.11 cm) were obtained on the MS medium supplemented with 1 mg/L BAP, when using nodal fragments as explants. For this type of explants, except for the concentration of 1 mg/L BAP, cytokinin supplementation of the culture medium had an inhibitory effect on the growth of shoots, an effect manifested by shorter lengths of shoots. The same trend was observed for the shoot tip explants, except for variants V2 (1 mg/L BAP) and V6 (2 mg/L 2iP) in which a higher average length of shoots was recorded compared to the control without growth regulators (Tab. 1).

Table 1. Influence of explant type, cytokinin type, and cytokinin concentration on the number of shoots/explant and the length of the shoots; values represent mean ±standard deviation

Cytokinin	Concentration (mg/L)	Shoot tips		Nodal fragments	
		no. of shoots/explant	length of shoots (cm)	no. of shoots/explant	length of shoots (cm)
–	–	3.76 ±0.25	4.88 ±1.47	4.95 ±0.13	6.99 ±0.88
BAP	1	5.27 ±0.51*	6.51 ±0.28*	7.12 ±0.33*	8.11 ±1.07
	2	2.98 ±0.25*	3.68 ±0.37*	3.13 ±0.16*	3.82 ±0.32*
	5	3.54 ±0.58	3.08 ±0.49*	3.17 ±0.21*	3.38 ±0.49*
2iP	1	3.33 ±0.06	3.79 ±0.56*	5.07 ±1.02	5.81 ±1.81
	2	3.90 ±0.36	5.32 ±0.02	5.30 ±0.50	6.52 ±0.97
	5	2.94 ±0.31*	3.15 ±0.43*	4.00 ±1.13	3.98 ±1.63*
KIN	1	2.88 ±0.03*	3.58 ±0.42*	3.08 ±0.47*	2.39 ±0.39*
	2	3.27 ±0.08	3.21 ±0.07*	3.28 ±0.61*	2.89 ±0.71*
	5	4.02 ±0.55	4.25 ±0.70	4.97 ±0.21	4.69 ±0.23*

* significance of the difference at $p < 0.05$

Fig. 1. Shoots of *Mentha piperita* L. regenerated *in vitro* on MS medium supplemented with 1 mg/L BAP. A. *In vitro* propagated shoots. B. *In vitro* rooted shoots

After four weeks, the regenerated shoots were transferred to a fresh culture medium that supported the regenerative processes by determining a good proliferation of shoots. From a qualitative point of view, the biological material resulting from the regeneration of the explants had normal morphology, without aspects of vitrification, necrosis, or callus differentiation (Fig. 1A). The rooting phase was not necessary, the shoots developing roots on the multiplication medium (Fig. 1B).

The acclimatization rate of *in vitro* plantlets was 96%. Peppermint regenerated *in vitro* has preserved the morphological characteristics of the mother stock plants (Fig. 2A, B).

Fig. 2. Peppermint regenerated *in vitro*, fortified in pots (A) and soil (B)

Multiplication of peppermint by conventional methods. Seed multiplication was also an efficient method of propagating peppermint, the seed germination rate one month after the start of the experiment was 98%.

Results obtained in biochemical analysis. The evaluation of the content of *free water*, *total water*, and *bound water* in plants showed higher values of these parameters in plants regenerated *in vitro* (V1) compared to those obtained by conventional method (V2). The average amount of free water was 82.62% in the case of variant V1, respectively 79.26% in the case of variant V2. On average, the total water content was 85.11% in regenerated plants *in vitro* and 81.19% in plants obtained by conventional method. The average amount of bound water was 2.49% in the case of variant V1, respectively 1.94% in the case of variant V2. The evaluation of the *dry matter content* of the analyzed plants showed higher values of vegetative mass in plants obtained by conventional method (18.81%) compared to those regenerated *in vitro* (14.89%) (Tab. 2).

The analysis of the *chlorophyll content* of the plants obtained by the two methods showed that, in the case of *in vitro* regenerated biological material (V1), both chlorophyll *a* and chlorophyll *b* recorded higher average values (1.51 mg chlorophyll *a*/g green substance, respectively 0.72 mg chlorophyll *b*/g green substance) compared to the values recorded in plants obtained by conventional method – V2 (1.08 mg chlorophyll *a*/g green substance, respectively 0.58 mg chlorophyll *b*/g green substance), Table 2.

The results of the quantitative evaluation of the *carotenoid pigments* in *Mentha piperita* L. also showed that, on average, the value obtained for *in vitro* regenerated plants (0.91 mg/g green substance) was higher than those obtained for plants regenerated by conventional method (0.67 mg/g green substance), Table 2.

The main indicators of chlorophyll assimilation in plants were also calculated, namely the chlorophyll *a*/*b* ratio, as well as the chlorophyll/carotene ratio. Both indicators showed the predominance of chloro-

Table 2. Evaluation of biochemical indicators in *Mentha piperita* L. plants regenerated by *in vitro* culture (V1) and conventional method (V2); values represent mean ± standard deviation

Biochemical indicators	V1	V2
Free water (%)	82.62 ±1.56	79.26 ±1.40
Total water (%)	85.11 ±1.17	81.19 ±1.29
Bound water (%)	2.49 ±0.39	1.94 ±0.13
Dry matter (%)	14.89 ±1.17	18.81 ±1.29
Chlorophyll *a* (mg/g green substance)	1.51 ±0.39	1.08 ±0.13
Chlorophyll *b* (mg/g green substance)	0.72 ±0.13	0.58 ±0.03
Chlorophyll *a*/*b*	2.08 ±0.15	1.87 ±0.15
Chlorophyll/carotene	2.47 ±0.05	2.48 ±0.10
Carotenoid pigments (mg/g green substance)	0.91 ±0.23	0.67 ±0.09
Total soluble carbohydrates (mg/g green substance)	136.55 ±7.68	138.62 ±8.47
Total polyphenols (mg TAE/g green substance)	0.10 ±0.01	0.25 ±0.02

phyll *a*, respectively of the chlorophyll pigments in the analyzed plants.

The method of obtaining the biological material did not have an important influence on the accumulation of *carbohydrates*, the values registered being similar in the samples from *in vitro* regenerated plants and in those from conventional cultures. On average, the soluble carbohydrate content was 136.55 mg/g green substance in plants regenerated *in vitro* (V1) and 138.62 mg/g green substance in plants obtained by conventional method (V2), Table 2.

In contrast, the *content of polyphenols* in peppermint plants was higher in the case of variant V2 (plants from conventional culture; 0.25 mg TAE/g green substance) compared to variant V1 (plants regenerated *in vitro*; 0.10 mg TAE/g green substance), Table 2.

DISCUSSION

One of the essential conditions for the successful initiation of *in vitro* cultures is to ensure asepsis. Our observations showed that the inclusion of peppermint in this culture system does not pose particular problems, the use of calcium hypochlorite for sterilization of biological material proved to be efficient.

In general, the regenerative potential of nodal fragments was superior to the regenerative potential of shoot tip explants. Our results are consistent with those reported by other authors who also obtained significantly higher proliferation efficiency of nodal explants compared to shoot tip explants [Ghanti et al. 2004, Raja and Arockiasamy 2009, Sarwar et al. 2009].

Supplementing the nutrient medium with 1 mg/L BAP induced an increase compared to the control of both the number of shoots/explant and the length of the shoots. Similar to our findings, Shelepova et al. [2021] reported that the MS medium supplemented with 0.5 mg/L BAP was the most optimal medium for the micropropagation of the peppermint cultivars studied. Cytokinins, especially BAP, were reported to overcome apical dominance, release lateral buds from dormancy, and promote the formation of shoots [George 1993]. The increase in BAP concentration led to a weaker morphogenetic response. Similar results have also been reported for *Mentha viridis* [Raja and Arockiasamy 2009], *Psoralea corylifolia* [Jeyakumar and Jayabalan 2000] and *Terminalia arjuna* Roxb. [Varghese et al. 2003].

The superior effect of BAP has been documented in *Mentha piperita* itself [Ghanti et al. 2004, Vaidya et al. 2019]. Similar results of BAP efficacy have also been reported for axillary proliferation in other *Mentha* species, such as *Mentha spicata* and *Mentha arvensis* [Hirata et al. 1990, Kukreja et al. 1991]. Contrary to these results, the superior effect of KIN compared to BAP has been documented in some studies even in

Mentha piperita [Nadaska et al. 1990, Sunandakumari et al. 2004].

The regenerated shoots have developed roots on the multiplication medium. This process of spontaneous root formation was also observed in the experiments performed by Shelepova et al. [2021], without the need for additional auxin treatment to induce rooting. Spontaneous formation of adventitious roots may be caused by root primordia and/or endogenous auxins present in the tissues of cultivated explants [de Klerk et al. 1999]. This is an advantage of *in vitro* multiplication, reducing the cost and production time of the propagating material by at least four weeks. In contrast to these results, Vaidya et al. [2019] reported the maximum number of roots (14.4) on the MS medium supplemented with 1.0 μM indolylbutyric acid (IBA).

In vitro plantlets were acclimatized in a proportion of 96%. Other authors also obtained survival rates of about 90–100% when acclimatizing *in vitro* regenerated plantlets of different *Mentha* species [Maity et al. 2011, Manik et al. 2012, Shelepova et al. 2021].

The scientific literature contains few studies that have performed a comparative evaluation of active constituents in plants multiplied by classical method and plants propagated *in vitro*. The results indicated that the regeneration method did not significantly influence the synthesis of these compounds [Phatak and Heble 2002, Rajasekharan et al. 2012, Shelepova et al. 2021]. Biochemical analyzes of total polyphenol content, flavonoid content, and Trolox Equivalent Antioxidant Capacity (TEAC) assay showed a higher level in the dried leaf extracts compared to the fresh leaf extracts, regardless of the method of propagation [Vaidya et al. 2019].

Our biochemical investigations have shown some differences between plants regenerated by the two methods. The content of photosynthetic pigments in plants regenerated *in vitro* was higher than in plants obtained by conventional method. The soluble carbohydrates content was similar in the plants regenerated by the two methods of multiplication. Instead, the total phenol content in plants obtained by classical method was higher than in plants regenerated *in vitro*. These results appear to be consistent with the theory of carbon nutrients, which states that in situations in which environmental conditions are favorable and plants have access to excess carbon and nutrients (as in *in vitro* culture), vegetative growth (primary metabolism) generally displays resource priority over secondary metabolism [Matyssek et al. 2005]. Contrary to our results, there is a report that indicated that plants obtained from tissue culture had a higher phenol content compared to plants grown in the field [Aliyu et al. 2012].

CONCLUSIONS

Micropropagation is an efficient method for the large-scale multiplication and preservation of *Mentha piperita* L. The application of the established protocol can ensure the stable production of this commercial crop thus meeting the global demand for peppermint biological material. Although biochemical investigations revealed some differences between *in vitro* regenerated plants and those obtained by conventional method, the results obtained show that micropropagation can be used successfully to obtain high-quality peppermint biological material, a potential source of bioactive compounds with therapeutic effect.

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