IMPROVEMENT OF THE *IN VITRO* DEVELOPMENT OF *Mentha piperita* L. AND *Mentha longifolia* (L.) Huds. VARIETIES

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Abstract. Peppermint varieties are representative regarding therapeutical properties. The aim of this paper was to study the *in vitro* germination and micropropagation potential of two mint cultivars, namely *Mentha piperita* and *M. longifolia. In vitro* germination of *Mentha longifolia* seeds has been stimulated with gibberellic acid. Using an experimental system with two stages of three months, we obtained 50 microshoots per culture from every variety. Two variants of the basal Murashige - Skoog medium supplemented with benzyl adenine (BAP), 1 - naphthylacetic acid and yeast extract have been optimal for *in vitro* microshoot multiplication of the both cultivars. The proportions of the growth factors differed, i.e. 5:1:100 mg/L for *Mentha piperita* and 3:0.5:100 mg/L for *M. longifolia*. The capacity of both mint cultivars to maintain their viability for a long period between transfer on a fresh culture medium was evident. We developed an *in vitro* conservation strategy and obtained an increased number of regenerants that can ensure a considerable amount of biomass to cover the required vegetal material useful in cosmetic and pharmaceutical industry.

Keywords: *Mentha longifolia in vitro* culture, microshoots, *Mentha piperita in vitro* growth factors, in vitro plant conservation, aromatic and medicinal plants.

Rezumat. Optimizarea dezvoltării *in vitro* la varietățile *Mentha piperita* L. și *Mentha longifolia* (L.) Huds. Scopul lucrării noastre a fost acela de a studia capacitatea de germinare *in vitro* și micropropagare la două varietăți ale genului *Mentha*, și anume *Mentha piperita* și *Mentha longifolia*. Germinarea semințelor de *Mentha longifolia* a fost stimulată cu acid giberelic. Utilizând un sistem experimental în două etape a câte trei luni, am obținut 50 microlăstari per cultură pentru fiecare varietate. S-au dovedit a fi optime două variante ale mediului bazal Murashige - Skoog, suplimentate cu benzil adenină, acid naftil acetic și extract de drojdie. Fiecare varietate a avut o evoluție favorabilă pe variante conținând concentrații diferite ale acestor factori de creștere. Pentru multiplicarea lăstarilor *Mentha piperita*, s-a dovedit a fi optim raportul 5: 1 : 100 mg/L de citokinină (BAP), auxină (ANA) și extract de drojdie iar pentru *Mentha longifolia*, raportul de 3: 0.5, 100 mg/L din acești elicitori. A fost evidentă capacitatea ambelor varietăți de a-și păstra viabilitatea pe o perioadă îndelungată de timp între efectuarea de subculturi pe mediu proaspăt. Am dezvoltat o metodologie de conservare *in vitro* si am obținut un număr mare de regeneranți ce asigură o cantitate de biomasă apreciabilă care să acopere necesarul de material vegetal util în industria cosmetică și farmaceutică.

Cuvinte cheie: cultura *in vitro* la *Mentha longifolia*, microlăstari, factori de creștere in vitro pentru *Mentha piperita*, conservarea *in vitro* la plante, plante aromatice și medicinale.

INTRODUCTION

The *Mentha* genus of the Lamiaceae Family comprises a wide range of varieties (Fig. 1; BRAHMI et al., 2017). Recent reviews analyse *Mentha piperita* L. as a therapeutic herb useful for producing nutraceutical, cosmetics and pharmaceuticals (HUDZ et al., 2023). GOMEZ et coworkers (2015) studied the *in vitro* production of plantlets and the capacity of multiplication of eight species of mint plant, namely *M. piperita, M. suaveolens, M. canadensis, M. longiflora, M. aquatica, M. arvensis, M. gracilis* and *M. spicata* by five successive 30-day subcultures. *Mentha longifolia* L. (Lamiaceae) contain a wide variety of volatile oils (OKUT et al., 2017) with main components such as the limonene and carvone both in stems (15.3 and 15.1 %, respectively) and leaves (5.8 and 7.9 %, respectively) (BERTOLI et al., 2011); the plantlets obtained *in vitro* have a content of bioactive constituents that is safer than in field mature plants. The total yield of essential oils can be increased with the addition of cytokinins to the basal *in vitro* culture medium (SANTORO et al., 2013). Biotechnologies as protoplast fusions between some cultivars of mint as peppermint (*Mentha piperita* L. cv Black Mitcham) and spearmint (*Mentha spicata* L. cv Native Spearmint) may be used to generate novel mint germplasm with high-quality oil and also resistance to verticillium wilt. (KRASNYANSKI et al., 1998). PGR treatment with indole acetic acid (IAA) and salicylic acid in *M. spicata, M. longifolia and M. piperita* increases the concentrations of some important volatile metabolites such as monoterpenes, sesquiterpenes, esters, ethers, ketones, phenols and carboxicyclic compounds (KUNDU et al., 2021).

Optimal concentrations of growth factors were added to the MS basal medium for the *in vitro* sprouting of the plant.

Different variants of the *in vitro* culture medium, fortified with growth factors in different proportions, have been used by scientists. In this context, SHARMA et al., 2019 obtained maximum sprouting, avoiding the callus phase on an MS medium supplemented with 3.0 mg/L BAP and 1.5 mg/L kinetin with an average number of shoots per explant of 20 ±0.5. SIDHU et coworkers (2019) obtained about 70-80 shoots per culture after 6 weeks when the MS medium contained 2.00-3.00 mg/L kinetin and 1.00 mg/L IAA. An equally good response was observed when kinetin was replaced with BAP (2-3 mg/L) and the IAA level was reduced to 0.25 mg/L in the medium. BAP and Kin are better plant growth regulators for shoot induction when cultured on an MS medium and the addition of NAA along with cytokinins enhanced the number of shoots (SARWAR et al., 2009). On the other hand, according to ZARKI & ELMTILI (2012), shoot tips have proven to be the most effective in shoots proliferation, with high proliferation rate (100 %) and shoot number (14 shoots per explants), using an MS medium supplemented with 0.5 mg/L BAP.

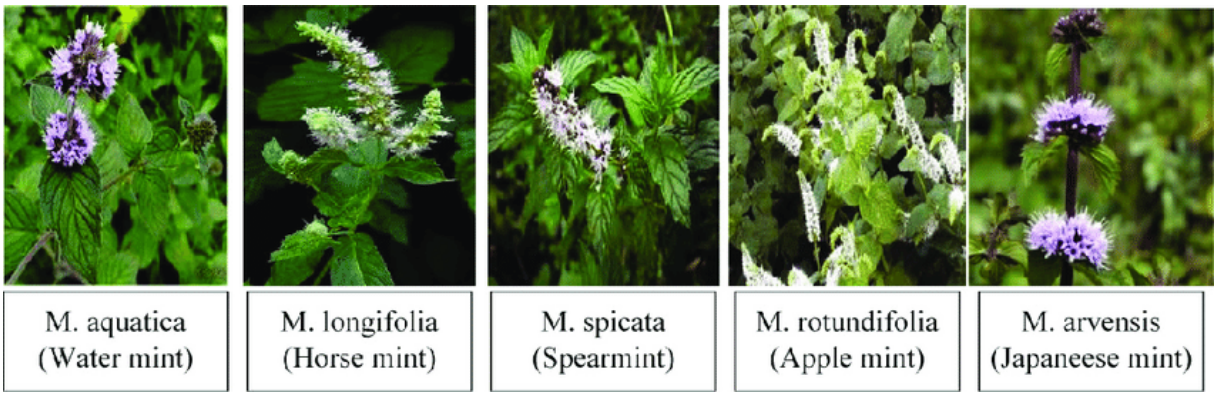

Figure 1. Varieties of the genus *Mentha* illustrated by BRAHMI et al., (2017).

MATERIAL AND METHODS

For the *in vitro* germination of *Mentha longifolia* (L.) Huds., we used seeds purchased from the online store www.semintelegumeflori.ro. For *Mentha piperita* L. *in vitro* multiplication, we used meristems sampled from the apical part of the plants purchased from the market place.

In order to fulfil aseptic conditions, seeds of *Mentha longifolia* and fragments of the apical stems of *Mentha piperita* mature plants have been washed previously with running tap water for 30 minutes, then, in the laminar flow bench, they have been submerged in a range of solutions of ethanol 70 % for 1 minute, mercuric chloride of 0.1 % for three minutes and rinsed with sterile bi-distilled water after these stages. The capacity of *in vitro* multiplication of these aromatic plants has been stimulated with the growth factors benzyl adenine (BAP), gibberellic acid and yeast extract as an elicitor added to the basal MS formula (Murashige - Skoog, 1962). The reactivity of in vitro vegetal inoculi has been tested in six variants of *in vitro* culture media, namely: I) MS supplemented with 1 mg/ L BAP and 1 mg/L gibberellic acid, II) MS with 1 mg/l BAP + 0.5 gibberellic acid, III) MS supplemented with 3 mg/L BAP and 1 mg/L gibberellic acid, IV) MS variant supplemented with 3 mg/L BAP + 0.5 mg/L NAA + 100 mg/L yeast extract, V) MS variant supplemented with 5 mg/L BAP + 1 mg/L NAA + 100 mg/L yeast extract VI) MS + 3 BAP + 0.5 IAA VII) MS + 1.5 IBA. The length and the number of regenerants per explant cultivated has been evaluated. In one variant used by us we modified the method of MANIK et al., (2012) by adding 100 ml yeast extract to the listed components. For germination, we used Petri dishes with a small diameter of 6 cm, then we transferred the shoots to Erlenmeyer dishes with a capacity of 500 ml, a height of 18 cm and a diameter of 6 cm. After inoculation, the *in vitro* culture dishes, in a series of ten per experiment, have been placed in the growth chamber.

RESULTS AND DISCUSSIONS

Mentha longifolia seeds started to germinate on basal Murashige - Skoog culture medium after one week; the germination of *Mentha longifolia* seeds reached a 80 % percentage; the differentiation of shoots started on a variant of nutritive culture medium with 1 mg/L gibberellic acid and 1 mg/L BAP; regarding the effect of growth regulation factors on the germination of seeds, ELHINDI et al., (2016) elaborated a successful methodology for overcoming seed dormancy and optimizing seed germination to peppermint by soaking seeds in gibberellic acid before inoculating them on the culture medium. With gibberellic acid, DHORAN & GUDADHE, 2012 increased seed germination by 47%. NAA has been reported to increase seed germination of *Mentha arvensis* by 32.3%.

In the subsequent stages we multiplicated shoots for *Mentha longifolia* on MS with 1 mg/L gibberellic acid and 3 mg/L BAP, then continued on MS variants with 3 mg/L BAP, 0.5 mg/L NAA and 100 mg/L yeast extract. The results of MANIK et al, 2012 revealed that a high shoot length is achieved with 3 mg/L BAP and 0.5 mg/L NAA. In the first three months, an active adventitious shoot formation was obvious for both species as in the experiments of ZAGORSKAYA & YEGOROVA, 2018, that used as *in vitro* stimulators of growth 1 mg/L BAP and 0.5 mg/L IAA. The *in vitro* response regarding shoot initiation was dependent on the culture media composition and the concentration of the added growth regulators. According to AKTER & HOQUE (2018), *in vitro* cultures require lower concentrations of BAP as 1 mg/L in order to be initiated and micropropagated. Assessing the effect of cytokinins and auxins on shoot development for *Mentha*, the results of previous researchers revealed that a good reactivity on media containing BAP (150µl/50ml) as cytokinin and NAA (20µl/50ml) as auxin can be obtained (KHAN et al., 2021). TULY et al. (2015) used BAP (1 mg/l) and NAA (1 mg/l) as growth regulators, for a shoot regeneration protocol, 55 days after inoculation, with an efficient *in vitro* response. MEHTA et al. (2012) mention that shoot multiplication *in vitro* for *Mentha piperita* has been accomplished by adding the growth regulator BAP in 2 mg/L concentration; 2.5 mg/L of BAP produces a significant number of shoots starting from the embryogenic callus of *Mentha spicata* (SAMANTARAY et al., 2012). 0.5 mg/L IBA (indolyl-3-butyric acid) induced lateral shoots (ŁYCZKO et al., 2020). SHELEPOVA et al., (2021) stimulated the development of numerous lateral shoots of *Mentha* cultivars using 0.5 mg/L BAP. SHARAN et al. (2014) also used a

higher quantity of BAP than NAA, obtaining adventitious bud regeneration from internode explants that can assure micropropagation of *Mentha* (Fig. 2a, b).

According to the results of ISLAM et al., (2017), a 3 mg/L BAP concentration stimulates the highest number of shoots per explants while the highest root proliferation is assured by 1.5 mg/L IBA added to the basal MS medium. *M. piperita* has been maintained in the *in vitro* culture in storage for at least six months on a slow growth 1/2 MS medium supplemented with 4.0% sorbitol (ZAYOVA et al., 2021). We obtained similar results to RANDOMIR et al. (2022) regarding some aspects of *in vitro* culture: the shoots of peppermint have the ability to develop roots along the shoots, and the rooting phase can be unnecessary. RADOMIR et al., 2022 obtained a high multiplication rate (7.12 shoots/explants) and a length of the shoots of (8.11 cm) on the MS medium supplemented with 1 mg/L BAP to *Mentha piperita*. Our regenerants of peppermint have been growing in length in Erlenmeyer dishes, reaching the length of the dish culture, of about 18 cm while *Mentha longifolia* reached a lengths of 12 - 15 cm (Figs. 3a, b and 4a, b). Clusters of shoots have a long beard aspect at *Mentha longifolia* (Fig. 3a). Every explant from the Erlenmeyer dish was surrounded by a bunch of lateral shoots (Fig. 3b) that in time became jointed in about 50 shoots. We used a subculturing period of three months. Images 3 - 5 presents different successive stages of inoculi development. Using an experimental system with two stages of three months, we obtained 50 microshoots per culture dish from every variety. Two variants of the basal Murashige - Skoog medium supplemented with benzyl adenine (BAP), 1-naphthylacetic acid and yeast extract have been optimal for *in vitro* microshoot multiplication of both cultivars. The proportions of the growth factors were different, namely 5:1:100 mg/L for *Mentha piperita* (Fig. 5) and 3: 0.5: 100 mg/L for *Mentha longifolia* (Fig. 3*). Mentha piperita* rooted and multiplicated on two variants of MS supplemented with 1.5 mg/L IBA, and MS + 3 BAP + 0.5 IAA (Fig. 6). Comparing the two different genotypes, the different colour shade was highlighted as light green for *Mentha longifolia* (Fig. 3) and dark green for *Mentha piperita* (Fig. 5); the thickness of the shoot stems also differed, which was stronger in the case of *Mentha piperita*. All the variants of the *in vitro* culture medium stimulated lateral shoot formation, but the variant with yeast extract stimulated the increase of the interval of subculturing and the number of lateral shoots. The capacity of both mint cultivars to maintain their viability for a long period between transfer on a fresh culture medium was obvious. The two varieties have *in vitro* micropropagation potential; all nutritive variants presented a positive effect on inducing shoot morphogenesis to *Mentha longifolia*; regarding *Mentha piperita*, the variant with the highest concentration of BAP and NAA beside yeast extract triggered the morphogenetic response in shooting development and multiplication, as well as the variant with cytokin BAP and indole acetic acid (3:0.5). We developed an *in vitro* conservation strategy and obtained an increased number of regenerants that can ensure a considerable amount of biomass to cover the required vegetal material useful in the cosmetic and pharmaceutical industry.

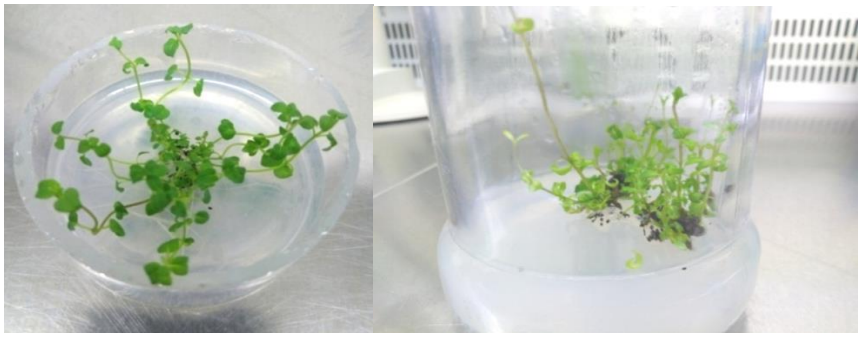

Figure 2a. Seeds of *Mentha longifolia* germinated on Murashige - Skoog;
b. shoots germinated and differentiated on MS, with the addition of BAP 1 mg/L and 1 mg/L gibberellic acid.

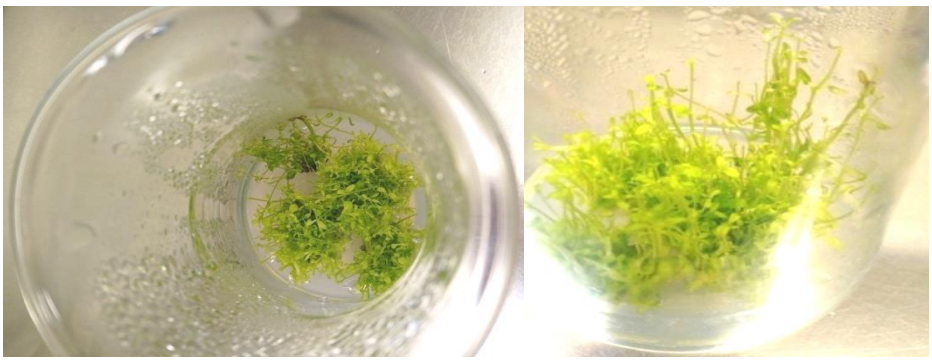

Figure 3a. Clusters of *Mentha longifolia* adventitious lateral shoots regenerated on MS supplemented with 3 mg/L BAP mg/L, 0.5 mg/L NAA and 100 mg/L yeast extract. b. light green bunch of *Mentha longifolia* regenerated shoots.

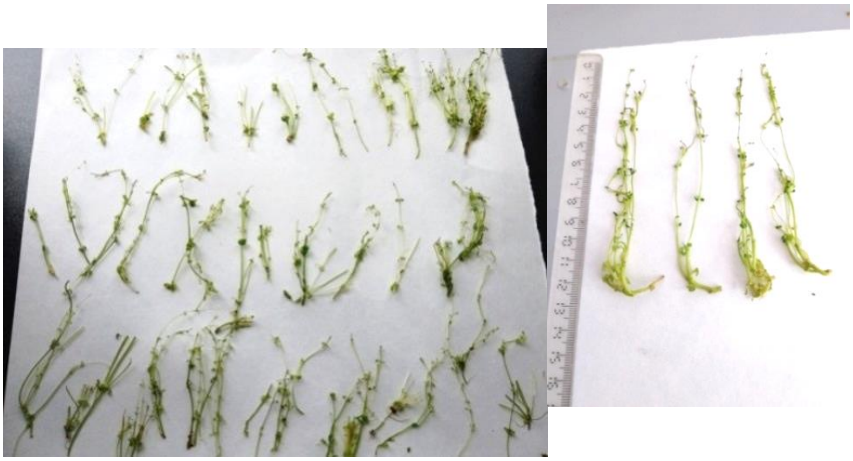

Figure 4a. 40 shoots of *Mentha longifolia* detached from the bunch.
b. morphometric parameters of *Mentha longifolia* shoots.

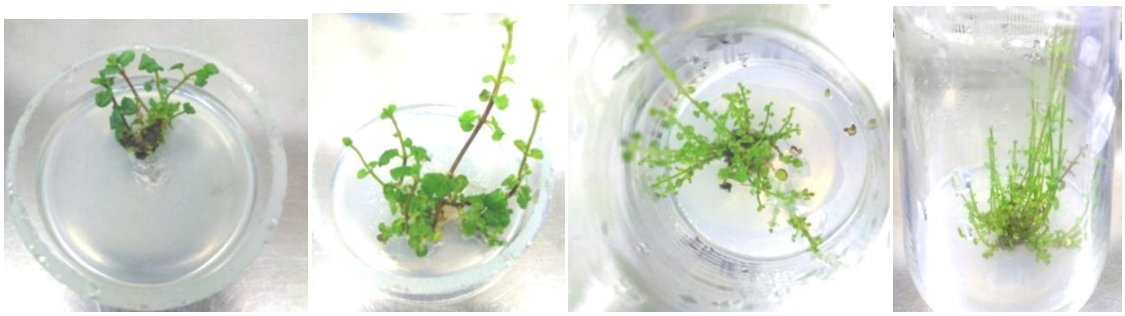

Figure 5. *Mentha piperita* shoots, differentiated on an MS variant, supplemented with 5 mg/L BAP + 1 mg/L NAA + 100 mg/L yeast extract.

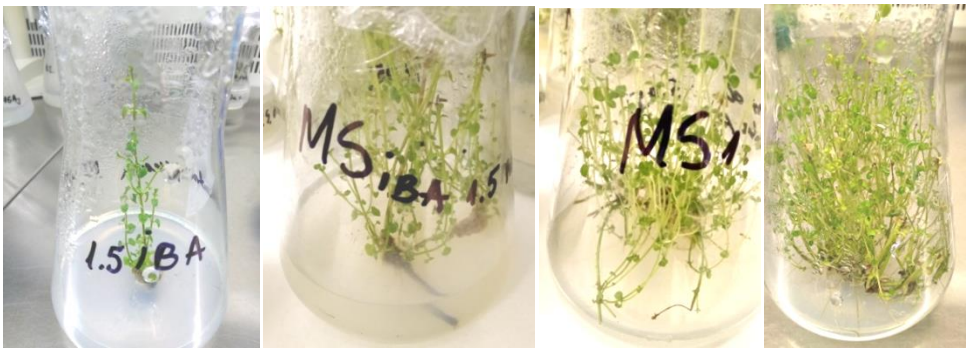

Figure 6. *Mentha piperita* shoots multiplicated on MS supplemented with 1.5 mg/L IBA and MS with 3 BAP + 0.5 IAA.

CONCLUSIONS

The shoot regeneration frequency depends on the culture medium variant used and the type of explant. The cultures of the *Mentha piperita* and *Mentha longifolia* mint varieties have the capacity to maintain their viability *in vitro* by subculturing at three months for a period of 12 months. The tissue cultures can be initiated from seeds for *Mentha longifolia* or fragments of the peppermint donor plant, stimulated with gibberellic acid, BAP, NAA and yeast extract and propagated for a long period on the optimal variants of MS. The protocol drawn up for medium term micropropagation can be applied to stimulate the obtaining of an appreciable amount of vegetal material used in the cosmetic and pharmaceutic industry.

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