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Menthol from the stem and leaf *in-vitro Mentha piperita* Linn.

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Abstract. The need for menthol in Indonesia is increasing annually, but it is not followed by increases the availability in the country, so the number of imports is increasing each year. Therefore, in this research conducted a study using plant tissue culture techniques to produce secondary metabolite especially menthol using shoot multiplication. A single node of *Mentha piperita* L. was inoculated on three kinds of growth medium, which are MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA, MS + 0.5 mg L^{-1} BA, and MS + 2 mg L^{-1} BA. Based on fresh weight and morphological observation, MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA was the best growth medium. Based on the previous experiment, the study was aiming to continue observing the concentration of menthol found in *M. piperita* shoot. Stems and leaves of *M. piperita* were dried in room temperature, the dried stems and leaves were extracted using steam distillation method with ratio 0.3 g of dried stems and leaves in 25 mL aquadest. The crude extract was analyzed by gas chromatography (HP 6890) using INNOWAX 19095N-123 column. The results showed that 2 wk was the best amount of time to obtain the highest concentration of menthol specifically (1 218.5 ± 47.1) mg L^{-1} (yield = 9.748 %).
Keywords: Gas chromatography, menthol, shoot multiplication, steam distillation

1. Introduction

Mentha spp. belongs to Labiatae family, there are three main species which have high economic value like *Mentha piperita* L. The industrial demands of *M. piperita* product are high, but Indonesia cannot fulfill the demands without import. In 2006 Indonesia imported *Mentha* spp. product worth the USD 3.78×10^6 [1]. *M. piperita* is a first sterile hybrid generation between *M. spicata* and *M. aquatica* [2]. The main compound from *Mentha* essential oil is menthol; this compound is produced in peltate glandular trichome which located at leaf [3].

The purpose of this research is to increase the production of menthol using plant tissue culture technique. Cytokinin is phytohormone that induced shoot proliferation and cell division [4]. Plant tissue culture is known to produce plants that grow faster [5]. In addition, the concentration of secondary metabolites, especially monoterpenes in *Lavandula pedunculata in vitro*, is known to be greater than plants grown in their natural [6]. The growth medium used for multiplication of shoots with the aim of increasing menthol concentration is Murashige & Skoog media (MS) + 6-Benzylaminopurine (BAP) + Naphthaleneacetic acid (NAA) [7]. The advantage of *in vitro* shoot multiplication techniques is that it is able to produce target compounds under controlled conditions from changes in weather and soil conditions, plants obtained free from microorganisms and insects, all types of plants can be used in this technique and reduce labor costs and increase productivity [8].

2. Materials and methods

2.1. Plant materials, treatment, and growing condition

This research was carried out at the Laboratory of Plant Biotechnology and Biopurification and Biomolecular Laboratory, Faculty of Biotechnology, University of Surabaya. The peppermint seeds (Biopot®) used in this study were pre-sterilized using bactericidal and fungicidal solution for an hour. The surface sterilization of explants was continued in Laminar Air Flow Cabinet with immersion in 70 % ethanol for 1 min, and 5.25 % NaOCl solution for 5 min followed by rinsing with sterile H2O at least three times. Furthermore, sterilization was carried out again by immersion in 1.75 % NaOCl solution for 10 min followed by rinsing with sterile water at least three times. Sterilized mint seeds are planted on MS media without hormones. The culture bottle was incubated under the white fluorescent lamps. Explants used are nodes from mint plants that are approximately 3 mo. The plant is cut to only one node. Furthermore, nodules cut into various growth media (table 1.) The culture was incubated in a culture room with a temperature of (25 ± 2) °C with the lighting of white fluorescent lamps and observed the formed tissue.

Table 1. Growth media variation.

Media
MS + 0.5 mg L^{-1} 2.4-D
MS + 1 mg L^{-1} 2.4-D
MS + 2 mg L^{-1} 2.4-D
MS + 4 mg L^{-1} 2.4-D
MS + 6 mg L^{-1} 2.4-D
MS + 0.5 mg L^{-1} NAA
MS + 2 mg L^{-1} NAA
MS + 4 mg L^{-1} NAA
MS + 6 mg L^{-1} NAA
MS + 8 mg L^{-1} NAA
MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} BAP
MS + 2 mg L^{-1} NAA + 1 mg L^{-1} BAP
MS + 2 mg L^{-1} NAA + 2 mg L^{-1} BAP
MS + 1 mg L^{-1} BAP
MS + 2 mg L^{-1} BAP
MS + 2 mg L^{-1} BAP + 1 mg L^{-1} NAA
MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP

2.2. Extraction and determination of menthol

The extraction samples used were leaves and stems of mint plants obtained from the bud multiplication process that have been dried in room temperature under air conditioning. The solvent used was 25 mL of water (distilled water) to extract 0.3 g of dried stems and *M. piperita* leaves, the sample was extracted using the steam distillation method. Then the extract obtained was analyzed for menthol content using a gas chromatography device (HP 6890) with INNOWAX 19095N-123 column [9]. For the ex vitro sample used stem and leaves of *M. piperita ex vitro* at 3 mo.

2.3.Data analyses

The research conducted was an experimental study in the laboratory with a Completely Randomized Design (CRD). In this study two non-parametric data were obtained because the data wasn't normally distributed, namely the best media used to grow *M. piperita* plants in vitro, and data on menthol production monitored every week. To determine the best growth media, and the best time to harvest, need to obtain the fresh weight of *M. piperita in vitro*, and menthol concentration for 7 wk. The

statistic test used in this research is the Kruskal-Wallis test and if significance found in the Kruskal-Wallis test continue using Dunn-Bonferroni as post hoc test.

3. Results and discussions

3.1. Preliminary test

Preliminary tests were carried out to determine the three types of growth media to be tested. Further, the aspects considered were morphology of *M. piperita* grown *in vitro*. From this preliminary test selected, MS medium + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA, MS + 0.5 mg L^{-1} NAA and MS + 2 mg L^{-1} NAA to be used at the next research stage because it has the best morphology. The reason for choosing MS media was 0.5 mg L^{-1} NAA and MS + 2 mg L^{-1} NAA compared to MS + 1 mg L^{-1} BA and MS + 2 mg L^{-1} BA,because the hormones NAA and IAA (Indole-3-Acetic Acid) were able to increase the oil content of essential oils and menthol in *M. piperita* [9]. Then MS + 0.5 mg L^{-1} NAA and 2 mg L^{-1} NAA has a better morphology than MS + 2 mg L^{-1} BA + 1 mg L^{-1} NAA, the morphology in question is the appearance of the plant (abnormal).

Table 2. Preliminary test result.

Media	Tissue/organ formed
MS + 0.5 mg L^{-1} 2.4-D	Callus
MS + 1 mg L^{-1} 2.4-D	Callus
MS + 2 mg L^{-1} 2.4-D	Callus
MS + 4 mg L^{-1} 2.4-D	Browning
MS + 6 mg L^{-1} 2.4-D	Browning
MS + 0.5 mg L^{-1} NAA	Shoot + callus
MS + 2 mg L^{-1} NAA	Shoot + callus
MS + 4 mg L^{-1} NAA	Browning
MS + 6 mg L^{-1} NAA	Browning
MS + 8 mg L^{-1} NAA	Browning
MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} BA	Shoot + callus
MS + 2 mg L^{-1} NAA + 1 mg L^{-1} BA	Browning
MS + 2 mg L^{-1} NAA + 2 mg L^{-1} BA	Browning
MS + 1 mg L^{-1} BA	Shoot + callus
MS + 2 mg L^{-1} BA	Shoot + callus
MS + 2 mg L^{-1} BA + 1 mg L^{-1} NAA	Shoot + callus
MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA	Shoot

3.2. Growth index curve

Growth Index Curve is obtained from the final weight of the stem and fresh leaves divided by fresh initial weight. The calculation of the growth index was done by weighing fresh initial weight before planting and after being cultivated for (1, 2, 3, 4, 5, 6 and 7) wk. Observation of the growth index stopped at week 7 because the index value had decreased compared to the 6th week.

Table 3. Fresh weight of *M. piperita* at 6 wk old.

Media	Fresh weight (g)
MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA	0.136 ± 0.071
MS + 0.5 mg L^{-1} NAA	0.129 ± 0.035
MS + 2 mg L^{-1} NAA	0.179 ± 0.121

The three growth media did not have a significant effect using the Kuskal-Wallis test at α = 0.05

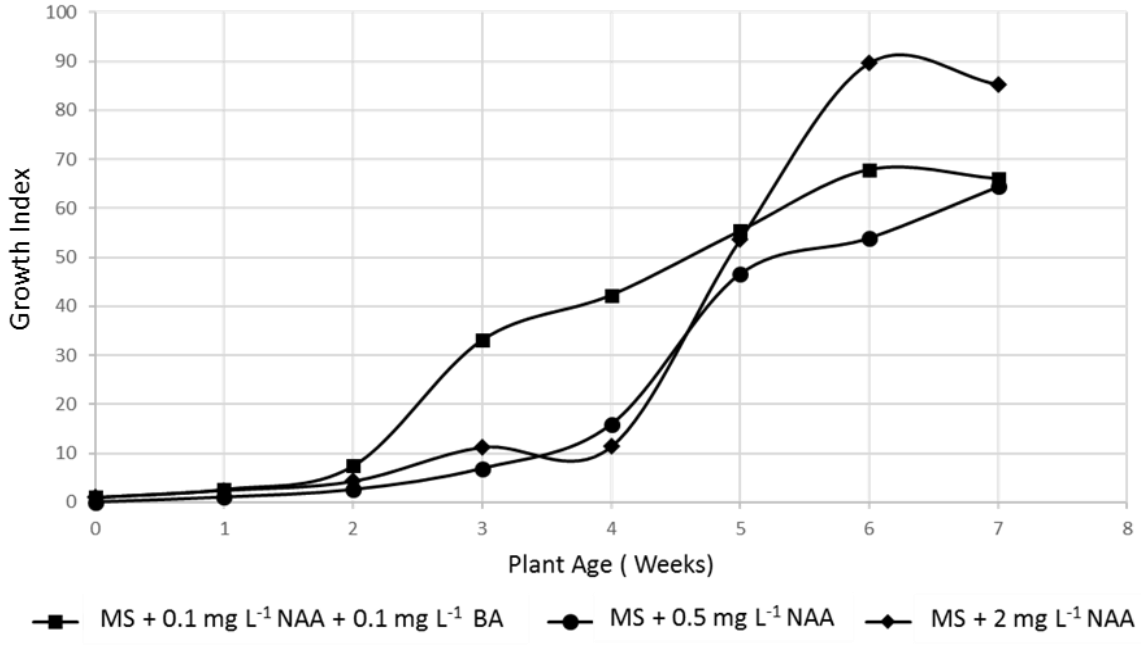

Figure 1. *M. piperita* growth index curve on MS media + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA, MS + 0.5 mg L^{-1} NAA and MS + 2 mg L^{-1} NAA.

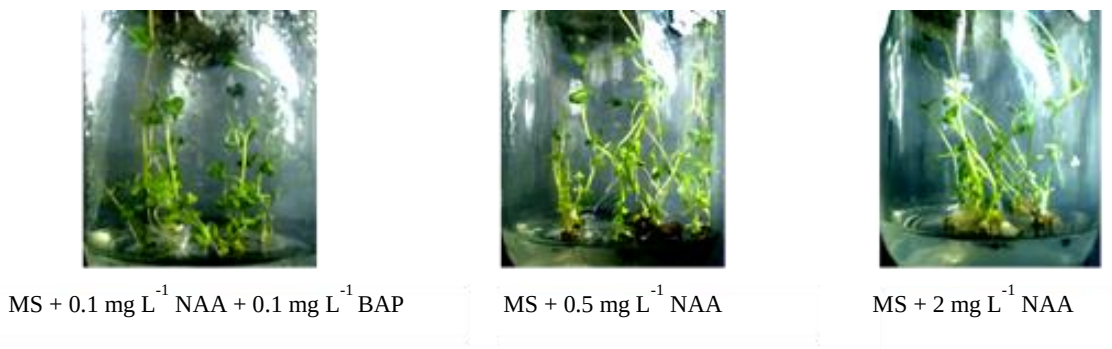

Figure 2. Morphology of *M. piperita in-vitro*.

Fresh weight of *M. piperita* grown on MS + 2 mg L^{-1} NAA is the largest among other growth media. This is due to the presence of callus at the base of the plant stem. Callus also found at the base of the plant stem that grown using MS + 0.5 mg L^{-1} NAA. *M. piperita* that grew at MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA obtained plants do not form callus and have a heavier mass than MS media + 0.5 mg L^{-1} NAA. Menthol compounds are stored in the peltate glandular trichome [3]. The peltate glandular trichome is found in parts of plants exposed to air [10]. So it can be said that the larger/, the heavier plant, the more peltate glandular trichome, in other words, the more menthol that can be extracted. In addition, other factors considered by the morphology of plants planted *in vitro*. There was no monoterpene found in Mentha spicata callus because the glandular oil gland or peltate glandular trichome had not been formed because callus cells were undifferentiated plant cells [11]. Therefore, the MS medium was selected + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA as the best growth medium.

3.3. Extraction and determination of menthol

Extraction of menthol compounds was carried out using the steam distillation method because it is commonly used to isolate essential oils. The distillation process is stopped until all the water in the flask has evaporated. From steam distillation results obtained ± 24 mL of crude menthol extract from steam distillation. The crude extract obtained was analyzed using a gas chromatography device (HP 6890) with the INNOWAX 19095N-123 column.

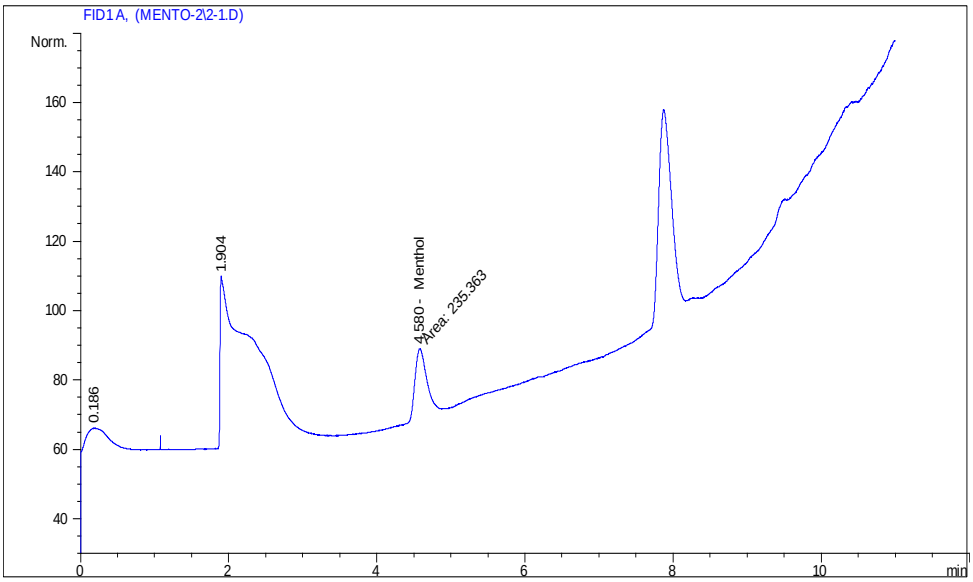

Figure 3. Profile of gas chromatography on crude extract *M. piperita* grown on MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA medium at 2 wk old.

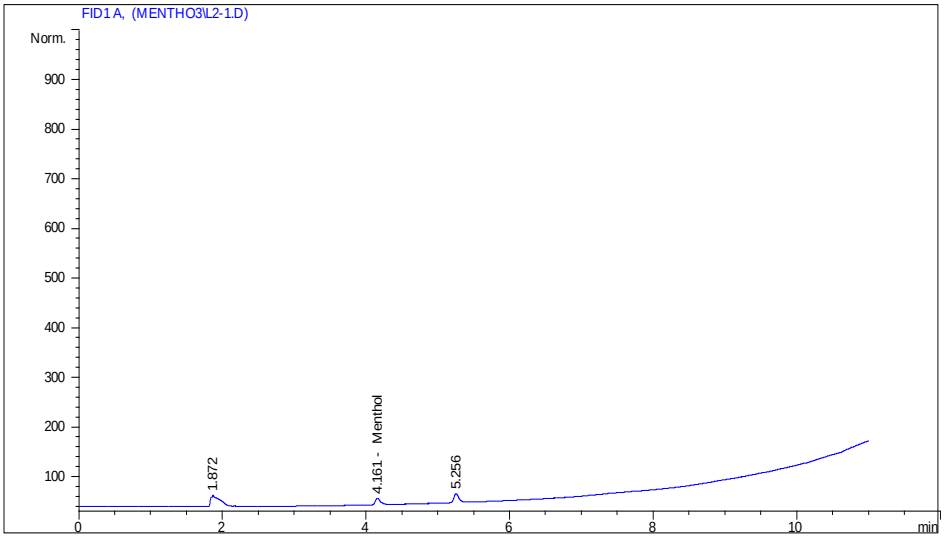

Figure 4. Profile of gas chromatography on crude extract *M. piperita* has grown on *ex-vitro*.

There is a difference between the crude extracts of *M. piperita in-vitro* and *ex-vitro*, the peaks that appeared at retention time was around 5 min in the crude extract profile of *ex-vitro* plants but were not found in crude plant extracts *in-vitro*. And the crude extracts of plants *in-vitro* found peaks at retention time about 8 min which were not found in crude extracts of *ex-vitro* plants. In other words, the *M. piperita* plant *in-vitro* is not capable of producing secondary metabolites that are exactly the same as their original plants and is able to form secondary metabolites that are completely different from the

original plants. Several factors that influence the difference in results is nutrition availability and environmental conditions. The availability of nutrients includes the concentration of sugar, nitrate, phosphate and growth regulating substances whereas environmental conditions include temperature, light intensity and pH [12]. Plant tissue culture systems are able to produce secondary metabolites that are completely different from the original plants because of very different environmental conditions, while the levels can be the same, larger or smaller [13]. *In-vitro* plant growth media have been given nutrition, sufficient growth regulating substances, and the growth environment is conditioned stable with adequate lighting, the temperature of 20 °C and pH of 5.8. All treatments given to plants *in-vitro* are common treatments to optimize growth in plant tissue culture while the *ex-vitro* plants all of these factors are not controlled at all, or in other words depending on nature.

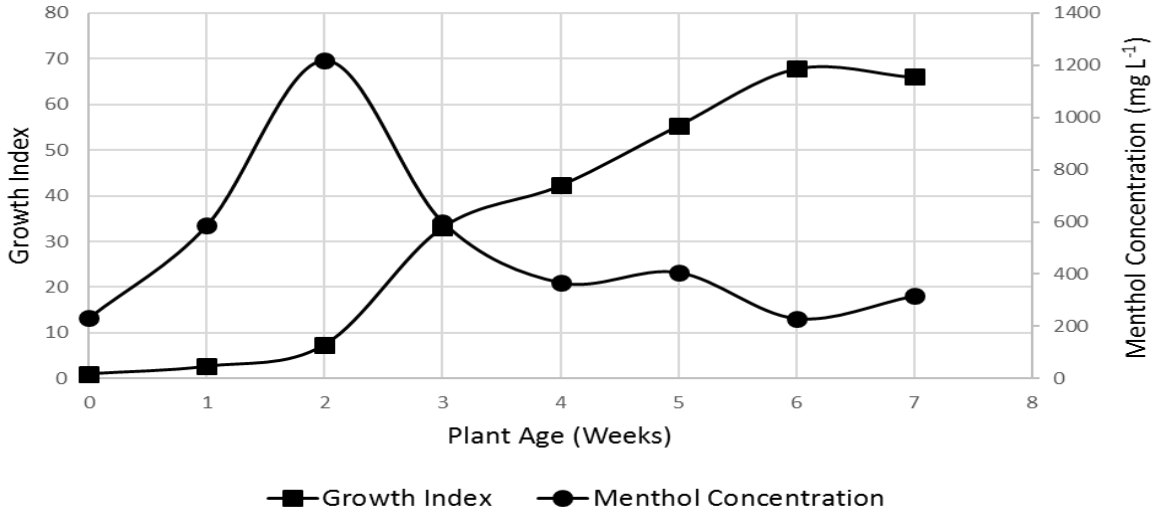

Figure 5. Menthol concentration curve and *M. piperita* growth index at 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA compared to incubation time

Table 4. Menthol concentration observed every week grown at MS + 0.1 mg L^{-1} BA + 0.1 mg L^{-1} NAA

Plant age (wk)	Menthol concentration (mg L^{-1})	Yield (% w/w)
0	$231.3^{ab} \pm 59.9$	1.85
1	$586.3^{ab} \pm 126.6$	4.69
2	$1\,218.5^{a} \pm 47.1$	9.748
3	$599^{ab} \pm 179$	4.792
4	$366.5^{ab} \pm 85.4$	2.932
5	$405.6^{ab} \pm 50.7$	3.245
6	$228.997^{ab} \pm 0.642$	1.832
7	$314.93^{ab} \pm 9.58$	2.519
Ex-vitro plant	$97.21^{b} \pm 1.373$	0.778

The different annotation followed by average value showed significance using Dunn-Bonferroni test $\alpha = 0.05$

The highest concentration of menthol was 2 wk old *M. piperita* that have grown using MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA, and the least concentration of menthol was *ex-vitro M. piperita*. This phenomenon is not in accordance with the theory, that the production of secondary metabolites generally occurs at the end of the stationary period when the nutrient supply is depleted [4]. This may be caused by menthol has been further metabolized to menthyl acetate [14]. There are also indications that the terpenoids used for defense have decreased due to the absence of threats.

Another factor that might cause the highest phenomenon of menthol concentration in the second week of culture is stress caused by injury at the beginning of cutting explants. This phenomenon was also showed on another research, where at 2 wk after the plant was injured there was a significant increase in the concentration of monoterpenes compared to non-injured plants [15]. This increase in the concentration of monoterpenes may be caused by an increase in the activity of the enzyme monoterpene cyclase [16]. Monoterpene cyclase plays a role in converting Geranyl pyrophosphate (GPP) to 4s-limonene [17]. GPP is the earliest compound in menthol biosynthesis pathway or it can be said that it is a compound that initiates menthol formation so that plants will tend to produce menthol in this condition. *M. piperita* grown in vitro is able to produce more menthol than *ex vitro*. This may be due to the availability of sufficient nutrients so that in vitro plants are able to produce more menthol. Besides that phytohormones are able to increase the levels of essential oil and menthol oil in *Mentha* spp. [18].

4. Conclusion

Based on this research, it can be concluded that the best growth medium for multiplying *M. piperita* shoots *in-vitro* was MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BA. Chromatogram profile on crude extracts of *M. piperita* plant in vitro showed three peaks, namely at about two retention times: 4.5 min and 8 min. Chromatogram profile on crude extract of *M. piperita ex-vitro* showed three peaks, namely at about two retention times: 4.5 min and 5 min. The menthol concentration observed for 7 wk in the largest *M. piperita* plant *in-vitro* was at the culture time of the 2nd week which was (1 218.5 ± 47.1) mg L^{-1} . The menthol concentration observed in the *M. piperita ex-vitro* plant was (97.21 ± 1.373) mg L^{-1} .

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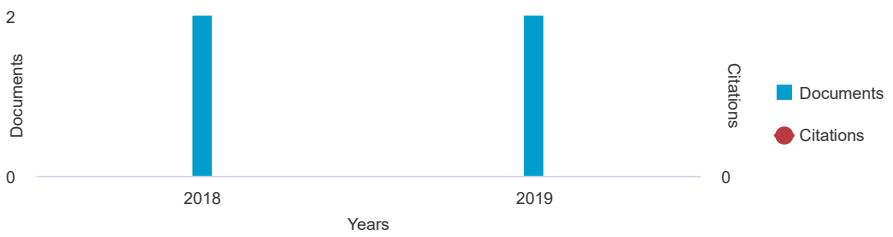

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Menthol from in vitro Stem and Leaf Mentha piperita

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Abstract. The need for menthol in Indonesia is increasing annually, but it's availability isn't enough to fulfill the need. Therefore, in this research we conducted a study using plant tissue culture techniques to produce secondary metabolite especially menthol using shoot multiplication. Single node of *Mentha piperita* were inoculated on three kinds of growth medium, which are MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP, MS + 0.5 mg L^{-1} NAA, and MS + 2 mg L^{-1} NAA. Based on fresh weight and morphological observation, MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP was the best growth medium. Based on the previous experiment, the study was aiming to continue observing the concentration of menthol found in *Mentha piperita* shoot. Stems and leaves of *Mentha piperita* were dried in room temperature, the dried stems and leaves were extracted using steam distillation method with ratio 0.3 g of dried stems and leaves in 25 mL aquadest. The crude extract was analyzed by gas chromatography (HP 6890) using INNOWAX 19095N-123 column. The results showed that two weeks was the best amount of time to obtain highest concentration of menthol specifically 1.218.5 ± 47.1 mg L^{-1} (yield = 9.748%).

Keywords: Gas chromatography, *Mentha piperita*, menthol, shoot multiplication, steam distillation

1. Introduction

Mentha spp. belongs to Labiatae family, there are three main species which are have high economic value like *Mentha piperita*. The industrial demands of *Mentha piperita* product is high but Indonesia cannot fulfill the demands without import. In 2006 Indonesia imported *Mentha* spp. product worth US $ 3,78 million [1]. *M. piperita* is first sterile hybrid generation between *M. spicata* and *M. aquatica* [2]. The main compound from *Mentha* essential oil is menthol, this compound is produced in peltate glandular trichome which located at leaf [3].

The purpose of this research is to increase the production of menthol using plant tissue culture technique. Cytokinin is phytohormone that induced shoot proliferation and cell division [4]. Plant tissue culture is known to produce plants that grow faster [5]. In addition, the concentration of secondary metabolites, especially monoterpenes in *Lavandula pedunculata* in vitro, is known to be greater than plants grown in their natural [6]. The growth medium used for multiplication of shoots with the aim of increasing menthol concentration is Murashige & Skoog media (MS) + 6-Benzylaminopurine (BAP) + Naphthaleneacetic acid (NAA) [7]. The advantage of in vitro shoot multiplication techniques is that it is able to produce target compounds under controlled conditions from changes in weather and soil conditions, plants obtained free from microorganisms and insects, all types of plants can be used in this technique, and reduce labor costs and increase productivity [8].

2. Materials and Methods

2.1. Plant Materials, treatment and growing condition

This research was carried out at the Laboratory of Plant Biotechnology and Biopurification and Biomolecular Laboratory, Faculty of Biotechnology, University of Surabaya. The peppermint seeds

(Biopot®) used in this study were pre-sterilized using bactericidal and fungicidal solution for an hour. The surface sterilization of explants was continued in Laminar Air Flow Cabinet with immersion in 70% ethanol for 1 minute, and 5.25% NaOCl solution for 5 minutes followed by rinsing with sterile H_2O at least three times. Furthermore, sterilization was carried out again by immersion in 1.75% NaOCl solution for 10 minutes followed by rinsing with a sterile water at least three times. Sterilized mint seeds are planted on MS media without hormones. The culture bottle was incubated under the white fluorescent lamps. Explants used are nodes from mint plants that are approximately 3 months old. The plant is cut to only one node. Furthermore, nodules cut into various growth media (Table 1.)The culture was incubated in a culture room with a temperature of 25 ± 2°C with the lighting of white fluorescent lamps and observed the formed tissue.

Table 1. Growth Media Variation

Media
MS + 0.5 mg L^{-1} 2,4-D
MS + 1 mg L^{-1} 2,4-D
MS + 2 mg L^{-1} 2,4-D
MS + 4 mg L^{-1} 2,4-D
MS + 6 mg L^{-1} 2,4-D
MS + 0.5 mg L^{-1} NAA
MS + 2 mg L^{-1} NAA
MS + 4 mg L^{-1} NAA
MS + 6 mg L^{-1} NAA
MS + 8 mg L^{-1} NAA
MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} BAP
MS + 2 mg L^{-1} NAA + 1 mg L^{-1} BAP
MS + 2 mg L^{-1} NAA + 2 mg L^{-1} BAP
MS + 1 mg L^{-1} BAP
MS + 2 mg L^{-1} BAP
MS + 2 mg L^{-1} BAP + 1 mg L^{-1} NAA
MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP

2.2. Extraction and determination of Menthol

The extraction samples used were leaves and stems of mint plants obtained from the bud multiplication process that have been dried in room temperature under air conditioning. The solvent used was 25 mL of water (distilled water) to extract 0.3 g of dried *Mentha piperita* stems and leaves, the sample was extracted using the steam distillation method. Then the extract obtained was analyzed for menthol content using a gas chromatography device (HP 6890) with INNOWAX 19095N-123 column [9]. For the ex vitro sample used stem and leaves of *Mentha piperita* ex vitro at 3 months old.

2.3. Data analyses

The research conducted was an experimental study in the laboratory with a Completely Randomized Design (CRD). In this study two non-parametric data were obtained because the data wasn't normally distributed, namely the best media used to grow *Mentha piperita* plants in vitro, and data on menthol production monitored every week. To determine the best growth media, and the best time to harvest, need to obtain fresh weight of *Mentha piperita* in vitro, and menthol concentration for seven weeks. The statistic test used in this research is Kruskal-Wallis test and if significance found in the Kruskal-Wallis test continue using Dunn-Bonferroni as post hoc test.

3. Results and Discussions

3.1. Preliminary Test

Preliminary tests were carried out to determine the 3 types of growth media to be tested further, the aspects considered were morphology of *Mentha piperita* grown in vitro. From this preliminary test selected, MS medium + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP, MS + 0.5 mg L^{-1} NAA and MS + 2 mg L^{-1} NAA to be used at the next research stage because it has the best morphology. The reason for choosing MS media was 0.5 mg L^{-1} NAA and MS + 2 mg L^{-1} NAA compared to MS + 1 mg L^{-1} BAP and MS + 2 mg L^{-1} BAP,because the hormones NAA and IAA (Indole-3-Acetic Acid) were able increase the oil content of essential oils and menthol in *Mentha piperita* [10]. Then MS + 0.5 mg L^{-1} NAA and 2 mg L^{-1} NAA has a better morphology than MS + 2 mg L^{-1} BAP + 1 mg L^{-1} NAA, the morphology in question is the appearance of the plant (abnormal).

Table 2. Preliminary test result

Media	Tissue/Organ Formed
MS + 0.5 mg L^{-1} 2,4-D	Callus
MS + 1 mg L^{-1} 2,4-D	Callus
MS + 2 mg L^{-1} 2,4-D	Callus
MS + 4 mg L^{-1} 2,4-D	Browning
MS + 6 mg L^{-1} 2,4-D	Browning
MS + 0.5 mg L^{-1} NAA	Shoot + callus
MS + 2 mg L^{-1} NAA	Shoot + callus
MS + 4 mg L^{-1} NAA	Browning
MS + 6 mg L^{-1} NAA	Browning
MS + 8 mg L^{-1} NAA	Browning
MS + 2 mg L^{-1} NAA + 0.5 mg L^{-1} BAP	Shoot + callus
MS + 2 mg L^{-1} NAA + 1 mg L^{-1} BAP	Browning
MS + 2 mg L^{-1} NAA + 2 mg L^{-1} BAP	Browning
MS + 1 mg L^{-1} BAP	Shoot + callus
MS + 2 mg L^{-1} BAP	Shoot + callus
MS + 2 mg L^{-1} BAP + 1 mg L^{-1} NAA	Shoot + callus
MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP	Shoot

3.2. Growth Index Curve

Growth Index Curve is obtained from the final weight of the stem and fresh leaves divided by fresh initial weight. The calculation of the growth index was done by weighing fresh initial weight before planting and after being cultivated for 1, 2, 3, 4, 5, 6 and 7 weeks. Observation of the growth index stopped at week 7 because the index value had decreased compared to the 6th week.

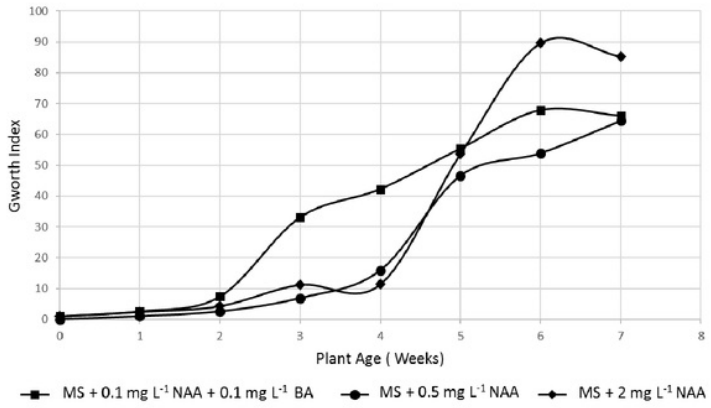

Figure 1. *Mentha piperita* growth index curve on MS media + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP, MS + 0.5 mg L^{-1} NAA and MS + 2 mg L^{-1} NAA.

Table 3. Fresh Weight of *Mentha piperita* at 6 weeks old.

Media	Fresh Weight (g)
MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP	0.136 ± 0.071
MS + 0.5 mg L^{-1} NAA	0.129 ± 0.035
MS + 2 mg L^{-1} NAA	0.179 ± 0.121

The three growth media did not have a significant effect using the Kuskal-Wallis test at $\alpha = 0.05$

Figure 2. Morphology of *Mentha piperita* in vitro.

Fresh weight of *Mentha piperita* grown on MS + 2 mg L^{-1} NAA is the largest among other growth media. This is due to the presence of callus at the base of the plant stem. Callus also found at the base of the plant stem that grown using MS + 0.5 mg L^{-1} NAA. *Mentha piperita* that grew at MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP obtained plants do not form callus and have a heavier mass than MS media + 0.5 mg L^{-1} NAA. Menthol compounds are stored in the peltate glandular trichome [3]. The peltate glandular trichome is found in parts of plants exposed to air [11]. So it can be said that the larger/the heavier plant, the more peltate glandular trichome, in other words, the more menthol that can be extracted. In addition, other factors considered by the morphology of plants planted in vitro. There was

no monoterpene found in *Mentha spicata* callus, because the glandular oil gland or peltate glandular trichome had not been formed because callus cells were undifferentiated plant cells [12]. Therefore, the MS medium was selected + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP as the best growth medium.

3.3. Extraction and Determination of Menthol

Extraction of menthol compounds was carried out using the steam distillation method because it is commonly used to isolate essential oils. The distillation process is stopped until all the water in the flask has evaporated. From steam distillation results obtained ± 24 ml of crude menthol extract from steam distillation. The crude extract obtained was analyzed using a gas chromatography device (HP 6890) with the INNOWAX 19095N-123 column.

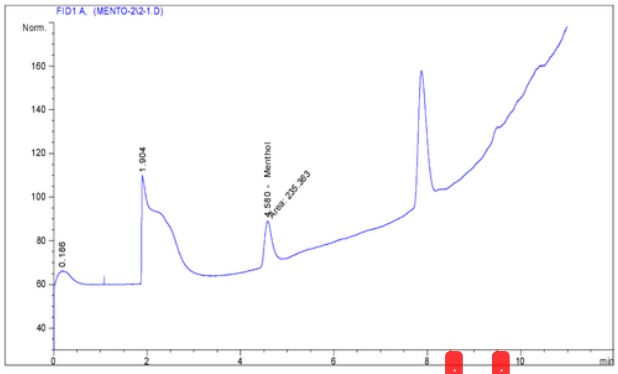

Figure 3. Chromatogram of crude extract *Mentha piperita* Grown on MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP medium at 2 weeks old.

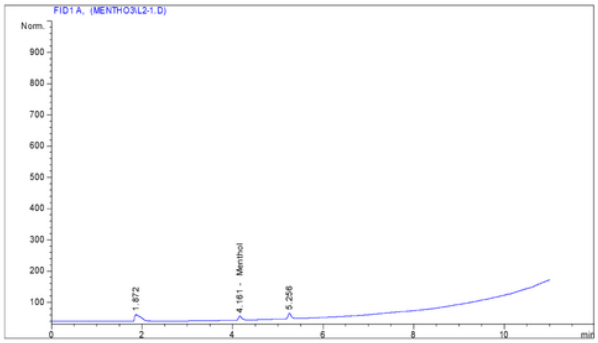

Figure 4. Chromatogram of crude extract *Mentha piperita* grown on ex vitro.

There is a difference between the crude extracts of *Mentha piperita* in vitro and ex vitro, the peaks that appeared at retention time were around 5 minutes in the crude extract profile of ex vitro plants but

were not found in crude plant extracts in vitro. And the crude extracts of plants in vitro found peaks at retention time about 8 minutes which were not found in crude extracts of ex vitro plants. In other words, the *Mentha piperita* plant in vitro is not capable of producing secondary metabolites that are exactly the same as their original plants and is able to form secondary metabolites that are completely different from the original plants. Several factors that influence the difference in results is nutrition availability and environmental conditions. The availability of nutrients includes the concentration of sugar, nitrate, phosphate and growth regulating substances. Whereas environmental conditions include temperature, light intensity and pH [13]. Plant tissue culture systems are able to produce secondary metabolites that are completely different from the original plants because of very different environmental conditions, while the levels can be the same, larger or smaller [14]. In vitro plant growth media have been given nutrition, sufficient growth regulating substances, and the growth environment is conditioned stable with adequate lighting, temperature of 20°C, and pH of 5.8. All treatments given to plants in vitro are common treatments to optimize growth in plant tissue culture. While the ex vitro plants all of these factors are not controlled at all, or in other words depending on nature.

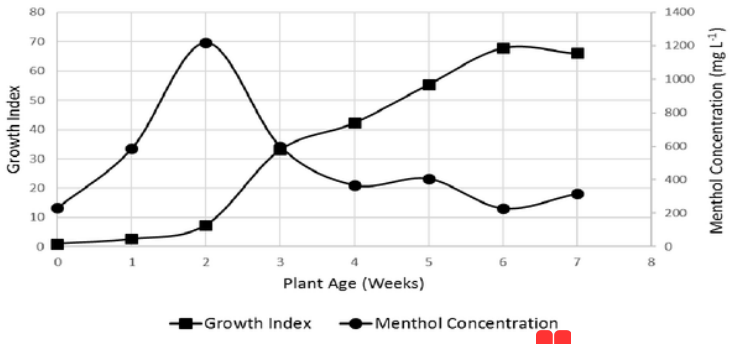

Figure 5. Menthol concentration curve and *Mentha piperita* growth index at 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BAP compared to incubation time

Table 4. Menthol concentration observed every week grown at MS + 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA

Plant Age (Weeks)	Menthol Concentration (mg L^{-1})	Yield (% w/w)
0	$231.3^{ab} \pm 59.9$	1.85
1	$586.3^{ab} \pm 126.6$	4.69
2	$1\,218.5^{a} \pm 47.1$	9.748
3	$599^{ab} \pm 179$	4.792
4	$366.5^{ab} \pm 85.4$	2.932
5	$405.6^{ab} \pm 50.7$	3.245
6	$228.997^{ab} \pm 0.642$	1.832
7	$314.93^{ab} \pm 9.58$	2.519
Ex vitro *plant*	$97.21^{b} \pm 1.373$	0.778

The different annotation followed by average value showed significance using Dunn-Befferoni test $\alpha = 0.05$

The highest concentration of menthol was 2 weeks old *Mentha piperita* that have grown using MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP, and the least concentration of menthol was ex vitro *Mentha piperita*. This phenomenon is not in accordance with the theory, that the production of secondary metabolites generally occurs at the end of the stationary period when the nutrient supply is depleted [4]. This may be caused by menthol has been further metabolized to menthyl acetate [15]. There are also indications that the terpenoids used for defense have decreased due to the absence of threats. Another factor that might cause the highest phenomenon of menthol concentration in the second week of culture is stress caused by injury at the beginning of cutting explants. This phenomenon was also showed on another research, where at 2 weeks after the plant was injured there was a significant increase in the concentration of monoterpenes compared to non-injured plants [16]. This increase in the concentration of monoterpenes may be caused by an increase in the activity of the enzyme monoterpene cyclase as occurs in *Abies grandis* plants [17]. Monoterpene cyclase plays a role in converting Geranyl pyrophosphate (GPP) to 4s-limonene [18]. GPP is the earliest compound in menthol biosynthesis pathway or it can be said that it is a compound that initiates menthol formation so that plants will tend to produce menthol in this condition. *Mentha piperita* grown in vitro is able to produce more menthol than ex vitro. This may be due to the availability of sufficient nutrients so that in vitro plants are able to produce more menthol. Besides that the hormones NAA and IAA (Indole-3-Acetic Acid) are able to increase the levels of essential oil and menthol oil in *Mentha piperita* [10]. There are another research showed that increasing BAP and sugar concentration on media didn't increase the production of menthol in *Mentha piperita* [19].

4. Conclusions

Based on this research, it can be concluded that the best growth medium for multiplying *Mentha piperita* shoots in vitro was MS + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} BAP. Chromatogram profile on crude extracts of *Mentha piperita* plant in vitro showed 3 peaks, namely at about 2 retention times: 4.5 and 8 minutes. Chromatogram profile on crude extract of *Mentha piperita* ex vitro showed 3 peaks, namely at about 2 retention times: 4.5 and 5 minutes. The menthol concentration observed for 7 weeks in the largest *Mentha piperita* plant in vitro was at the culture time of the 2nd week which was 1 218.5 ± 47.1 mg L^{-1}. The menthol concentration observed in the *Mentha piperita* ex vitro plant was 97.21 ± 1.373 mg L^{-1}.

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