



Effect of plant growth regulators in micropropagation of (*Mentha piperita* L.) plant In vitro

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Received: May 27, 2025	Abstract This study was conducted on one of the most important medicinal plants with significant pharmaceutical value, namely <i>Mentha piperita</i> . The aim was to propagate peppermint using tissue culture techniques and combinations of plant growth regulators to obtain pathogen-free plants in large numbers. The study was carried out in the Tissue Culture Laboratory, College of Agriculture, University of Karbala, during the fall season of 2024, and included several experiments. The first experiment investigated the effect of NaOCl at different concentrations and exposure times on sterilizing seeds used as explants. The second experiment evaluated different concentrations of growth regulators at the initiation stage. The third experiment examined two types of cytokinins at different concentrations during the multiplication stage. The sterilization experiment showed that treatment with 2% NaOCl for 15 minutes achieved the lowest contamination rate (0%) without affecting explant viability. In the second experiment, BA at 1 mg L ⁻¹ resulted in the highest response rate (82.50%), while NAA at 0.1 mg L ⁻¹ gave the best response among auxin treatments (66.66%). In the third experiment, BA at 2 mg L ⁻¹ was superior in producing the highest shoot number, length, and fresh and dry weights of the vegetative growth, which were 34.02 branches plant ⁻¹ , 9.06 cm, 13.65 mg, and 3.85 mg, respectively. Regarding the effect of Kin, the concentration of 1 mg L ⁻¹ gave the highest values for the number and length of shoots and their fresh and dry weights, which were 25.70 branches plant ⁻¹ , 7.72 cm, 13.19 mg, and 3.15 mg, respectively.
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Introduction

Mentha piperita is a medicinal and aromatic plant of the genus *Mentha*, belonging to the Lamiaceae family. It is a hybrid resulting from the crossing of two species, water mint (*M. aquatica* L.) and spearmint (*M. spicata* L.) [1]. It is a perennial herbaceous plant that thrives in moist conditions and grows in tropical and subtropical climates. Its original habitat is the Mediterranean basin, from where it spread to different

parts of the world [2]. The plant ranges in height from 30–90 cm. It has ribbed stems that are distinguished by their reddish color. The leaves are simple, opposite, serrated at the edges, and dark green in color. The flowers are borne in spike-shaped inflorescences, are whitish-purple, and consist of four sepals, four petals, and four stamens. The stigma has an obtuse end [3]. Several studies have indicated the multiple medicinal and industrial uses of peppermint, whether as a raw herb (dry or fresh) or as an essential oil, which is rich in bioactive compounds with therapeutic effects, particularly against respiratory diseases. The essential oil contains various terpene compounds, the most abundant of which is menthol, an active ingredient used as a topical pain reliever for migraines and muscle pain. It is also used in the treatment of congestion, cough, and colds, and as a fever reducer and bronchodilator. Peppermint exhibits antioxidant activity due to its high content of phenolic compounds, which suppress free radicals that cause health damage in humans, including leukemia, lung cancer, and stomach cancer. The methanolic extract of peppermint has also shown efficacy against HIV-1 due to its flavonoid content, which possesses antioxidant, antibacterial, and antiviral properties [4,5]. Furthermore, the aqueous extract of peppermint has been applied in food preservation as a natural alternative to synthetic preservatives, while its essential oil has demonstrated strong insecticidal activity, making it an environmentally friendly insecticide that is safe for humans [6]. Plant tissue culture is one of the fundamental techniques of plant biotechnology, serving as an alternative method for vegetative propagation. It has been applied to many plant species to achieve rapid propagation and year-round production by controlling environmental conditions and enhancing the accumulation of secondary metabolites. Tissue culture has also been used in genetic improvement programs and in the production of true-to-type plants to address several propagation-related challenges [7].

Plant growth regulators influence vital physiological and morphological processes of plant growth and development. Cytokinins, in particular, play a central role in enhancing shoot proliferation and increasing the levels of secondary metabolites [8]. The most commonly used cytokinins in tissue culture are benzyladenine (BA) and kinetin (Kin), which are essential in cell division, stimulating the production of DNA and RNA through the activation of proteins and genes responsible for enzyme formation. Studies have also shown that cytokinins stimulate the movement and translocation of nutrients within plant tissues and organs, in addition to promoting cell division and differentiation, breaking apical dominance, increasing chlorophyll content, delaying senescence, and enhancing shoot initiation and multiplication [9,10]. This technology has also proven effective in stimulating the production of secondary metabolites. The present study aimed to employ tissue culture technology in the micropropagation of peppermint and to evaluate the efficiency of different plant growth regulators.

Materials and Methods

This study was conducted in the Plant Tissue Culture Laboratory of the Department of Horticulture and Landscape Engineering, College of Agriculture - University of Kerbala in the fall of 2024.

Seed sterilization and germination experiment

The seed germination process was carried out after washing the seeds with water for 30 minutes. They were then transferred to a laminar air flow cabin previously sterilized with 70% alcohol. The seeds were surface sterilized with sodium hypochlorite (NaOCl) at an initial concentration of 6%, from which different working concentrations (0, 1, 2, and 4%) were prepared by dilution, with 100 ml for each treatment. Sterilization was performed for three time periods (5, 10, and 15 minutes), respectively. After sterilization, the seeds were washed three times with sterile distilled water under continuous stirring to remove traces of NaOCl.

Seed inoculation was then carried out in sterile culture tubes containing bridges made of sterile filter paper, partially immersed in Murashige and Skoog (MS) medium [11] supplemented with plant growth regulators. Mint seeds were placed on the filter paper bridges at a rate of 10 replicates for each concentration. The cultures were incubated in a growth chamber under a light intensity of 1000 lux with a photoperiod of 16 hours light and 8 hours dark, at a temperature of 23 ± 2 °C. Results were recorded 14 days after planting, based on the percentage of contamination.

Initiation experiment

Based on the results of the sterilization experiment, the best treatment was selected, and plant parts were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of BA (0, 1, and 1.5 mg L⁻¹) in combination with NAA (0 and 0.1 mg L⁻¹). Each treatment was replicated ten times. The cultures were incubated in the growth room at 23 ± 2 °C under 1000 lux illumination with a photoperiod of 16 h day⁻¹. The response percentage was recorded four weeks after planting.

Vegetative multiplication experiment

Based on the results obtained from the initiation stage, the best treatment was selected, and vegetative shoots were used. They were cut into 1 cm segments and cultured on Murashige and Skoog (MS) nutrient medium supplemented with BA at concentrations of 0, 1, 2, and 3 mg L⁻¹ in combination with KIN at 0, 0.5, 1, and 1.5 mg L⁻¹, together with a fixed concentration of NAA at 0.1 mg L⁻¹. Each treatment was replicated ten times. The plantlets were maintained under the same culture conditions as described previously. Data on shoot number, shoot length, and fresh and dry weights of vegetative growth were recorded after six weeks of culture.

Statistical analysis

The results were statistically analyzed using a Completely Randomized Design (CRD) with the SAS software package [12], and the treatment means were compared using the least significant difference (LSD) test at the 0.05 probability level.

Results and Discussion

Effect of NaOCl concentrations and sterilization period on the percentage of contamination of peppermint seeds

Table (1) shows the effect of sodium hypochlorite (NaOCl) concentrations and sterilization periods on the contamination percentage of peppermint seeds. The control treatment recorded 100% contamination at all exposure times. With increasing NaOCl concentration, the contamination rate decreased significantly, reaching 46.67%, 26.67%, and 3.33% at 1%, 2%, and 4% NaOCl, respectively. Increasing the sterilization period to 15 min also reduced the contamination rate to 30.00%, compared with 57.50% at 5 min. The interaction between NaOCl concentrations and sterilization periods had a significant effect in reducing seed contamination. However, it was observed that exposure to 4% NaOCl for 10 or 15 min delayed or completely inhibited germination, and produced small white seedlings devoid of chlorophyll. In contrast, treatment with 2% NaOCl for 15 min resulted in the lowest contamination rate (0.0%) without negatively affecting seed germination.

The effectiveness of sodium hypochlorite as a sterilizing agent can be attributed to the formation of hypochlorous acid (HOCl), which is a strong oxidizing compound with high efficiency in surface sterilization. This acid is formed when chlorine dissolves in water, as shown in the following equation [13]:

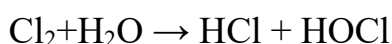


Table (1): Effect of sodium hypochlorite concentrations and sterilization periods on the contamination percentage of peppermint seeds after 14 days of culture on MS medium.

Time	NaOCl con.				Mean
	0	1	2	4	
5	100	70.00	50.00	10.00	57.50
10	100	50.00	30.00	00.00	45
15	100	20.00	00.00	00.00	30
L.S.D _(0.05)	10.88				5.44
Mean	100	46.67	26.67	3.33	
L.S.D _(0.05)	6.28				

The effect of BA and NAA concentrations on the percentage of plant initiation

Table (2) shows the effect of the growth regulators cytokinins and auxins on the emergence of plants, where the effect of BA was clear in increasing the response rate, as the concentration of 1 mg L⁻¹ significantly exceeded the rest of the concentrations

and gave the highest response rate of 82.5%. However, when the concentration of BA increased, it caused a decrease in the response rate, which is what happened at the concentration of 1.5 mg L^{-1} , which recorded a response rate of 65%, while the control treatment achieved the lowest rate of 30%. The results of the same table also showed that NAA was superior at the concentration of 0.1 mg L^{-1} , significantly achieving the highest rate of 66.66% compared to the control treatment, which achieved the lowest rate of 51.66%. As for the effect of the interaction between the concentrations of cytokinins and auxins on the response rate, the concentration of 1 mg L^{-1} BA in combination with 0.1 mg L^{-1} NAA achieved the highest response rate of 90%, while the control treatment achieved the lowest response rate of 20%.

Table (2): Effect of BA and NAA concentrations on the response of the plant part to initiation (%) after four weeks of culture on MS medium for peppermint.

BA con. mg l^{-1}	NAA con. mg l^{-1}		Mean
	0	0.1	
0	20	40	30
1	75	90	82.5
1.50	60	70	65
L.S.D _(0.05)	20.90		11.51
Mean	51.66	66.66	
L.S.D _(0.05)	9.39		

Effect of BA and Kin concentrations on the average number of shoots

Table (3) shows that BA was significantly superior at a concentration of 2 mg L^{-1} , giving the highest number of branches, which amounted to $34.02 \text{ branches plant}^{-1}$, while the lowest number was recorded in the control treatment, which amounted to $11.27 \text{ branches plant}^{-1}$. The same table also indicated that Kin was significantly superior at a concentration of 1 mg L^{-1} , which gave the highest response of $25.10 \text{ branches plant}^{-1}$ compared to the control treatment, which gave the lowest response of $17.44 \text{ branches plant}^{-1}$. Regarding the effect of the two-way interaction, the concentration of 2 mg L^{-1} BA in combination with 1 mg L^{-1} Kin was superior, giving the highest number of branches ($38.30 \text{ branches plant}^{-1}$), while the treatment that achieved the lowest number of branches was $6.30 \text{ branches plant}^{-1}$.

Table (3): Effect of BA and Kin concentrations on the number of branches (branches plant^{-1}) of peppermint after six weeks of culture on MS medium.

BA con. mg l^{-1}	Kin con. mg l^{-1}				Mean
	0.0	0.5	1.0	1.5	
0	6.30	10.75	12.66	15.35	11.27
1	14.37	16.50	20.75	18.60	17.56
2	28.60	33.14	38.30	36.05	34.02
3	20.50	22.14	28.70	25.16	24.13

L.S.D (0.05)	1.73				0.86
Mean	17.44	20.63	25.10	23.79	
L.S.D (0.05)	0.86				

Effect of BA and Kin concentrations on the length of vegetative shoot

The results of Table (4) show that there were significant differences in the shoot length of peppermint plants. BA was superior at a concentration of 2 mg L⁻¹, producing the longest shoots, which reached 9.06 cm. Regarding the effect of Kin concentrations, 1 mg L⁻¹ was significantly superior, reaching 7.72 cm, which did not differ significantly from 0.5 mg L⁻¹, which reached 6.08 cm, while the control treatment achieved the lowest shoot length of 4.92 cm. As for the effect of the interaction between BA and Kin on shoot length, the combination of 2 mg L⁻¹ BA with 1 mg L⁻¹ Kin achieved the highest shoot length, reaching 12.30 cm, while the lowest shoot length (2.87 cm) was recorded in the medium free of growth regulators.

Table (4): Effect of BA and Kin on the average length of vegetative branches (cm) of peppermint after six weeks of culture on MS medium.

BA con. mg l⁻¹	Kin con. mg l⁻¹				Mean
	0.0	0.5	1.0	1.5	
0	2.87	3.20	3.55	3.40	3.26
1	4.60	5.37	5.65	4.88	5.13
2	5.67	8.75	12.30	9.50	9.06
3	6.54	7.00	9.40	6.44	7.32
L.S.D (0.05)	1.50				0.75
Mean	4.92	6.08	7.72	6.05	
L.S.D (0.05)	0.75				

Effect of BA and Kin on fresh weight

Data in Table (5) indicate that there were significant differences when adding BA at different concentrations to the nutrient medium, as 2 mg L⁻¹ BA was significantly superior, recording the highest fresh weight of 17.84 mg. The results also indicated that Kin was significantly superior at a concentration of 1 mg L⁻¹, achieving the highest weight of 13.19 mg. As for the effect of the interaction between BA and Kin concentrations, it is noted from the results of the table that BA at 2 mg L⁻¹ in combination with Kin at 1 mg L⁻¹ was significantly superior, achieving the highest fresh weight of 19.85 mg, while the control treatment recorded the lowest weight of 0.80 mg.

Table (5): Effect of BA and Kin on the fresh weight of vegetative growths (mg) of peppermint plant after 6 weeks of cultivation on MS medium.

BA con. mg l ⁻¹	Kin con. mg l ⁻¹				Mean
	0.0	0.5	1.0	1.5	
0	3.28	5.30	6.01	7.45	5.51
1	7.10	8.25	10.38	9.35	8.77
2	14.28	18.52	19.85	18.12	17.84
3	10.15	12.09	16.53	14.89	13.42
L.S.D (0.05)	1.34				0.65
Mean	8.70	11.04	13.19	12.60	
L.S.D (0.05)	0.65				

The effect of BA and Kin on the dry weight

Table (6) shows that BA at a concentration of 2 mg L⁻¹ was significantly superior, giving the highest dry weight of 3.85 mg, while the lowest dry weight was recorded in the control treatment (1.62 mg). The results of the same table also showed that Kin was significantly superior at a concentration of 1 mg L⁻¹, achieving the highest dry weight of 3.15 mg, which did not differ significantly from 1.5 mg L⁻¹, which reached 3.10 mg. The lowest dry weight in Kin treatments was recorded in the control treatment, which reached 2.33 mg. As for the effect of the interaction between BA and Kin concentrations, it is noted from the results of the table that BA at 2 mg L⁻¹ in combination with Kin at 1 mg L⁻¹ was significantly superior, achieving the highest dry weight of 4.17 mg, while the control treatment recorded the lowest dry weight of 0.10 mg.

Table (6): Effect of BA and Kin concentrations on the dry weight (mg) of peppermint after six weeks of culture on MS medium.

BA con. mg l ⁻¹	Kin con. mg l ⁻¹				Mean
	0.0	0.5	1.0	1.5	
0	1.10	1.28	1.74	2.35	1.62
1	2.05	2.30	3.01	2.71	2.54
2	3.46	3.82	4.17	3.95	3.85
3	2.72	3.15	3.66	3.38	3.23
L.S.D (0.05)	0.85				0.43
Mean	2.33	2.64	3.15	3.10	
L.S.D (0.05)	0.43				

The results in the emergence stage showed the superiority of BA in increasing the response rate of peppermint plants. This may be due to the stimulating action of BA in inducing cell division and development. In addition, cytokinins help in indirect division, followed by cell division and stimulation of vegetative bud growth from

meristematic tissues, as well as the synthesis of RNA, DNA, enzymes, and proteins [15]. These results agreed with those reported by [16].

The results of Tables (3–6) generally indicated the superiority of BA in the studied traits, which included the number and length of shoots and the fresh and dry weights of peppermint plants compared to the control treatment. This superiority may be attributed to the stimulating action of BA in inducing cell division and development. It may also be due to the role of cytokinins as stable compounds that do not easily decompose and their high efficiency in breaking apical dominance. They enhance the development of xylem and phloem vessels and prevent chlorophyll degradation, thus stimulating cell division and increasing nucleic acid production, in addition to their role in attracting soluble and solid nutrients [17,18]. Cytokinins also play an important role in nutrient metabolism, carbon assimilation, and chloroplast development, which is positively reflected in the increase in the fresh and dry weights of vegetative growths [19]. The superiority of BA in vegetative characteristics may also be attributed to the fact that its side chain contains three double bonds, making it more active than other cytokinins, unlike Kin, which contains two bonds as well as a benzyl ring. For this reason, BA had a greater role in the multiplication of vegetative branches compared to Kin [20]. High levels of cytokinins, however, led to a decrease in growth rates due to hormonal imbalance within plant tissues, which impeded growth. This decrease does not necessarily mean cell death but usually inhibits growth [21]. These results are consistent with those reported by [22] when propagating peppermint (*Mentha piperita* L.) in vitro and by [23] when micropropagating the *Mentha* plant.

The results of this study indicated the possibility of employing plant tissue culture technology for the propagation of medicinal plants in large numbers within short periods of time and free of pathogens. The study also indicated the possibility of enriching the nutrient medium with different concentrations of cytokinins to stimulate axillary and lateral buds and obtain large numbers of vegetative branches. However, high and inappropriate concentrations of cytokinins negatively affected most of the studied traits. It is therefore recommended to use 1 mg L^{-1} Kin in combination with 2 mg L^{-1} BA per liter to achieve the best results in vegetative growth. Further studies should also be conducted on the economic feasibility of extracting the active ingredients using tissue culture techniques and comparing them with traditional cultivation methods.

This study demonstrated that BA, especially at 2 mg L^{-1} , was most effective in enhancing shoot number, length, and biomass of peppermint. The combination of 2 mg L^{-1} BA with 1 mg L^{-1} Kin produced the best vegetative growth. However, high or unbalanced levels of cytokinins reduced growth due to hormonal imbalance. Tissue culture thus offers a reliable method for producing pathogen-free peppermint plants on a large scale within a short period.

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