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RESEARCH PAPER

A study on tissue culture methods for stem segments of six mint varieties

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Abstract

Mint (*Mentha* spp.) is one of the world's major spice crops. Intraspecific and interspecific hybridization, along with artificial cultivation, have resulted in complex and diverse germplasm materials. To rapidly obtain genetically stable, sterile regenerated mint seedlings and establish a foundation for genetic transformation in mint germplasm improvement, this study used bud-bearing stem segments of the hybrid mint variety 'Msu' as explants. Through plant tissue culture methods, the effects of different hormone ratios and other conditions on plant regeneration were investigated. The results showed that surface sterilization of 'Msu' stem segments with 2% NaClO for 17 minutes achieved the best disinfection effect, with a sterility rate of 91.6%. The hormone combination of 1.0 mg/L 6-BA and 0.05 mg/L NAA in MS medium significantly induced axillary bud growth, achieving an induction rate of 100%. Additionally, 0.1 mg/L NAA effectively promoted adventitious root formation, with a rooting rate of 100%. Applying these optimized conditions to the tissue culture of five other mint varieties successfully produced regenerated seedlings, demonstrating broad applicability.

Keywords

Hormone, Mint, Rapid Propagation, Tissue Culture

Introduction

Mentha is a highly versatile medicinal herb and one of the world's major spice plants (Wang et al. 2003; Guo et al. 2023; Sun et al. 2023). Taxonomically classified in the Lamiaceae family, it is a perennial herb with multi-value attributes in food, medicine, and horticulture. Mint is rich in bioactive chemical components such as volatile oils (e.g., menthol, menthone) and flavonoids (e.g., quercetin) (Wu et al. 2019; Ramzi et al. 2022; Huang et al. 2024) endowing it with antibacterial, anti-inflammatory, analgesic, antipruritic, and antioxidant activities (Hua et al. 2018; Wang et al. 2020). Through deep processing technologies, diverse industrial application systems have been established: in pharmaceuticals, compound antibacterial preparations and neuralgia patches have been developed (Azmi et al. 2021; Li et al. 2022); in cosmetics, cooling and antipruritic functional skin care products have emerged (Han and Kim 2024) and in the food industry, it is used as natural preservatives and for innovative functional cool-flavored foods (Tian et al. 2020; Jangi et al. 2022).

The rich germplasm resources of mint form the cornerstone of its industrial applications. However, widespread intraspecific and interspecific hybridization in *Mentha* species, along with numerous cultivated varieties globally, has led to mixed sexual reproduction progeny and morphologically complex germplasm during introduction and exchange, resulting in heterogeneous genetic backgrounds that affect quality stability. Meanwhile, plant tissue culture has emerged as a critical technique for seedling propagation and variety improvement in recent years, owing to its advantages of rapid reproduction, season-independent growth, maintenance of elite genetic traits, and applicability for genetic transformation (Xu et al. 2017).

Reports have shown that stem, leaf, and petiole explants of mint can all induce differentiation and achieve plant regeneration under suitable conditions (Liu and Hao 2011; Zhang et al. 2013; Tang and Tian 2022; Xian et al. 2023; Ren et al. 2024; Jiang and Peng 2025). Therefore, establishing a simple, efficient, and universal tissue culture regeneration system for mint to rapidly propagate plants is of scientific and practical significance for maintaining elite trait stability, preventing germplasm degradation, and facilitating variety promotion and large-scale production. Current reports on mint tissue culture predominantly focus on a few varieties, lacking systematic exploration of tissue culture methods for diverse mint materials (Wu et al. 2018). This study conducted tissue culture experiments using domestic and foreign mint varieties with distinct aromas, employing axillary bud-bearing stem segments as explants to establish an efficient and universal rapid propagation method by comparing the effects of different hormones and their ratios on plant regeneration.

Materials and methods

Experimental materials

Six mint accessions previously collected and identified by our laboratory were used: 'Msu' (*Mentha suaveolens* × *piperita*), 'Mgr1' (*M. gracilis*), 'Mgr2' (*M. gracilis*), 'Mlo' (*M. longifolia*), 'Msp1' (*M. spicata* cv. 'English'), and 'Msp2' (*M. spicata* cv. 'Spear'). These plants were cultivated in a greenhouse under conditions of 20–25 °C, 6500 lx light intensity, and a 14 h/day photoperiod. For tissue culture, materials were maintained in a plant tissue culture room at 25 °C, 4200 lx light intensity, and a 12 h/day photoperiod.

The following reagents were used: MS (Murashige and Skoog) medium, 6-BA (6-benzylaminopurine), NAA (naphthaleneacetic acid), and agar (purchased from Phytotech Laboratories, USA); Timentin (acquired from Beijing Coolab Scientific Co., Ltd.); and NaClO, sucrose, and other chemicals (supplied by Sinopharm Chemical Reagent Co., Ltd.).

Obtaining sterile explants

Hybrid mint 'Msu' stem segments with axillary buds, selected for their diverse genetic background, were subjected to surface sterilization using NaClO solutions at varying concentrations (2% and 4%) and treatment durations (15, 17, and 19 min) to evaluate sterilization efficacy. Each treatment combination was replicated three times, with five culture bottles per replicate and 3–4 stem segments per bottle.

Detailed procedure

Upper stems with leaves from mint plants were excised and trimmed into 5–7 cm segments, then rinsed under running water for 10 min. Then leaves were removed, and stems were cut into 1–2 cm segments bearing axillary buds. Next in a laminar flow hood, segments were soaked in sterile distilled water for 1–2 min, surface-disinfected with 75% ethanol under agitation for 2 min, and rinsed 1–2 times with sterile distilled water. After ethanol removal, segments were sterilized with NaClO under agitation, followed by 4–5 rinses with sterile distilled water. Then segments were dried on sterile filter paper and inoculated onto MS medium. After 7 days of light incubation, sterilization rate (number of contamination-free explants / total inoculated explants × 100) and mortality rate (number of surviving explants / total inoculated explants × 100) were calculated. Using the optimal sterilization protocol identified for 'Msu', the same procedure was applied to 'Mgr1', 'Mgr2', 'Mlo', 'Msp1', and 'Msp2'.

Bud induction

MS medium served as the basal medium, supplemented with 10 g/L sucrose as the primary carbon source and 100 mg/L Timentin to suppress endophytic bacteria. Different combinations of cytokinin (6-BA) and auxin (NAA) were added to evaluate their effects on plant regeneration from 'Msu' stem segments with axillary buds. Surface-sterilized stem segments bearing axillary buds were inoculated onto induction media with varying hormone combinations. After 10 days of culture, the axillary bud regeneration rate (number of regenerated buds / total buds × 100) was calculated. Each treatment was replicated three times, with five culture bottles per replicate and 3–4 stem segments per bottle. The optimal hormone combination identified for 'Msu' was subsequently applied to 'Mgr1', 'Mgr2', 'Mlo', 'Msp1', and 'Msp2' using the same protocol.

Rooting culture

Half-strength MS medium (1/2 MS) was used as the basal medium, supplemented with 10 g/L sucrose and 100 mg/L Timentin, along with varying concentrations of 6-BA and NAA to assess their effects on root induction in regenerated shoots of 'Msu'. When regenerated shoots reached 2–3 cm in height, they were aseptically excised and transferred to rooting media with different hormone combinations. After 14–21 days of culture, the rooting rate (number of rooted plantlets / total plantlets × 100) was recorded. Each treatment included three replicates with five bottles per replicate and 2–3 shoots per bottle. The optimal rooting medium for 'Msu' was then tested on 'Mgr1', 'Mgr2', 'Mlo', 'Msp1', and 'Msp2'.

Data analysis

Data were analyzed using SPSS 27.0 software. One-way analysis of variance (ANOVA) was performed to assess statistical significance, followed by Duncan's multiple range test for post-hoc comparisons. Significant differences ($P < 0.05$) were denoted by distinct lowercase letters in tables and figures.

Results and analysis

Effects of NaClO concentration and treatment duration on surface sterilization of mint stem segments

Sodium hypochlorite (NaClO) is commonly used for surface sterilization of explants in plant tissue culture. Excessive NaClO concentrations or prolonged treatment durations can inhibit the growth of meristematic tissues (e.g., buds) and even cause browning and necrosis, thereby compromising regeneration capacity. Conversely, insufficient sterilization may fail to eliminate surface microorganisms, leading to bacterial or fungal contamination that exploits nutrients in the culture medium, forming colonies and hyphae, ultimately preventing tissue regeneration. To determine the optimal NaClO concentration and treatment duration for sterilizing mint stem explants, 'Msu' bud-bearing stem segments were first treated with 75% ethanol for 2 min, followed by immersion in 2% or 4% NaClO solutions for 15, 17, or 19 min. After inoculation on MS medium for 7 days, the sterilization rate (absence of contamination) and mortality rate (browning-induced death) of the stem segments were recorded.

As shown in Table 1, increasing NaClO concentration or extending treatment duration reduced contamination rates but simultaneously elevated browning mortality. Balancing contamination control and explant viability, the optimal protocol was identified as treatment with 2% NaClO for 17 min, achieving both low contamination rates and minimal browning-induced mortality.

Table 1. Effects of different NaClO concentrations and treatment times on 'Msu' stem segments.

Group	Concentration of NaClO (%)	Treatment time (min)	Infection rate (%)	Brownification rate (%)
1	2	15	14.4 ± 1.3a	6.4 ± 2.1b
2	2	17	8.4 ± 1.2b	7.2 ± 2.3b
3	2	19	7.7 ± 2.3bc	16.5 ± 2.5a
4	4	15	6.6 ± 2.4bc	16.8 ± 3.4a
5	4	17	4.8 ± 2.1bc	17.4 ± 2.3a
6	4	19	3.5 ± 1.9c	19.6 ± 2.2a

Note: Different lowercase letters indicate significant differences ($P < 0.05$).

Effects of hormone ratios on axillary bud induction

The differentiation and regeneration of plant tissues are primarily induced by two hormones: cytokinin and auxin. The optimal concentrations and ratios of these two hormones vary depending on specific factors such as species and explant type. To investigate the effects of different cytokinin (6-BA) and auxin (NAA) combinations on axillary bud regeneration in stem segment explants of 'Msu', the plant material was cultured on media with varying 6-BA:NAA ratios (designated as M1–M24; see Table 2). The results in Table 2 demonstrate: When 6-BA concentration was fixed at 1 mg/L, the axillary bud induction rate decreased from an initial 100% (Group M1) to approximately 58.5% (Group M4) as NAA concentration increased from 0.05 mg/L to 0.6 mg/L, but subsequently rose to 76.7% (Group M6) when NAA concentration further increased to 1 mg/L. Similar initial decline followed by recovery trends were observed with 6-BA concentrations of 1.5 mg/L and 2 mg/L across different NAA ratios, though the maximum induction rates of 71.7% (Group M7) and 66.7% (Group M13) remained lower than that of M1. When 6-BA concentration increased to 2.5 mg/L, Groups M19–M24 showed higher induction rates compared to Groups M7–M12 and M13–M18. The optimal induction rate reached 91.7% at a 20:1 6-BA:NAA ratio. However, despite the higher induction rate in M19 medium compared to M1, the regenerated stems and leaves exhibited excessive compactness and stunted growth (Fig. 1) due to the elevated 6-BA concentration. The combination of 1.0 mg/L 6-BA and 0.05 mg/L NAA (M1 medium) achieved 100% bud induction (Table 2).

As exemplified in Fig. 1, axillary buds cultured on M1 medium exhibited optimal growth, with intact leaf morphology, vibrant green coloration, and stems of moderate

Table 2. Effects of different hormone combinations on axillary bud regeneration in 'Msu'.

Medium	6-BA (mg/L)	NAA (mg/L)	Induction rate (%)
M1	1	0.05	100.0 ± 0.0a
M2	1	0.2	96.7 ± 1.1a
M3	1	0.4	66.7 ± 2.1abcdef
M4	1	0.6	58.5 ± 1.4bcdef
M5	1	0.8	75.1 ± 1.1abcde
M6	1	1	76.7 ± 2.4abcd
M7	1.5	0.075	71.7 ± 0.9abcdef
M8	1.5	0.3	70.0 ± 2.2abcdef
M9	1.5	0.6	33.3 ± 1.4f
M10	1.5	0.9	41.7 ± 1.7def
M11	1.5	1.2	46.7 ± 1.9cdef
M12	1.5	1.5	66.7 ± 1.9abcdef
M13	2	0.1	66.7 ± 1.3abcdef
M14	2	0.4	58.8 ± 2.0bcdef
M15	2	0.8	56.7 ± 3.1bcdef
M16	2	1.2	36.7 ± 1.6ef
M17	2	1.6	60.2 ± 1.7bcdef
M18	2	2	63.3 ± 3.2abcdef
M19	2.5	0.125	91.7 ± 1.12ab
M20	2.5	0.5	85.2 ± 1.1abc
M21	2.5	1	58.3 ± 1.2bcdef
M22	2.5	1.5	76.7 ± 2.4abcd
M23	2.5	2	80.0 ± 2.0abcd
M24	2.5	2.5	85.2 ± 1.3abc

Note: Different lowercase letters indicate significant differences ($P < 0.05$).

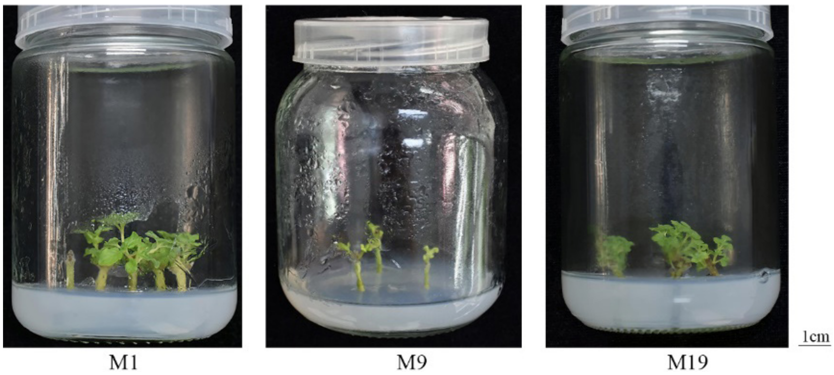

Figure 1. Axillary bud induction of 'Msu' stem segments in M1, M9, and M19 media.

length and thickness. In contrast, M9 medium showed suboptimal efficacy in axillary bud induction due to its hormone ratio, while M19 medium, despite yielding a higher number of induced buds, produced smaller leaves and shorter stems, resulting in overall inferior growth compared to M1. Therefore, the optimal medium for axillary bud induction was determined to be MS + 1 mg/L 6-BA + 0.05 mg/L NAA.

Effects of hormone concentrations on rooting

Regenerated shoots of 'Msu' (2–3 cm in height) were transferred to rooting media supplemented with varying concentrations of NAA and 6-BA (designated as R1–R12). Rooting status was assessed after 21 days of culture. As shown in Table 3, higher rooting rates and greater root numbers were observed in media without 6-BA and with low NAA concentrations (0.1 mg/L and 0.3 mg/L). These results indicate that root initiation in 'Msu' regenerated shoots is effectively promoted by low NAA concentrations, whereas the addition of 6-BA or elevated NAA levels reduces rooting rates and inhibits root differentiation.

As shown in Fig. 2, R1 (0 mg/L 6-BA, 0.1 mg/L NAA) and R2 (0 mg/L 6-BA, 0.3 mg/L NAA) exhibited higher root numbers and elevated rooting rates. After rinsing the root medium from regenerated plantlets, seedlings with well-developed root systems were observed to grow into robust plants directly in the culture bottles (Fig. 3). In contrast, at higher concentrations of 6-BA and NAA, such as in R11 (0.5 mg/L 6-BA, 0.3 mg/L NAA), rooting rates were significantly reduced, with seedlings displaying stunted root systems, sparse lateral roots, and indistinct primary roots. Therefore, the optimal medium for shoot rooting was determined to be 1/2 MS + 0.1 mg/L NAA.

Table 3. Effects of different hormone combinations on rooting of 'Msu'.

Medium	6-BA (mg/L)	NAA (mg/L)	Root count (piece)	Rooting rate (%)
R1	0	0.1	7 ± 2	100.0 ± 0.0a
R2	0	0.3	4 ± 2	91.7 ± 0.1a
R3	0	0.5	5 ± 1	83.3 ± 0.2a
R4	0.1	0.1	2 ± 2	25.2 ± 0.2b
R5	0.1	0.3	5 ± 2	22.0 ± 0.1b
R6	0.1	0.5	5 ± 2	16.7 ± 0.1b
R7	0.3	0.1	3 ± 2	27.3 ± 0.4b
R8	0.3	0.3	2 ± 2	12.5 ± 0.2b
R9	0.3	0.5	3 ± 2	8.3 ± 0.1b
R10	0.5	0.1	1 ± 1	14.5 ± 0.2b
R11	0.5	0.3	1 ± 1	8.3 ± 0.1b
R12	0.5	0.5	2 ± 2	6.7 ± 0.1b

Note: Different lowercase letters indicate significant differences ($P < 0.05$).

Regeneration of five other mint varieties

Based on the surface sterilization treatment of nodal stem segment explants and the optimal media for axillary bud induction and rooting established for the hybrid mint 'Msu', tissue culture regeneration experiments were conducted on five other mint varieties with distinct aromatic characteristics: 'Mgr1' and 'Mgr2' (Asian mint materials),

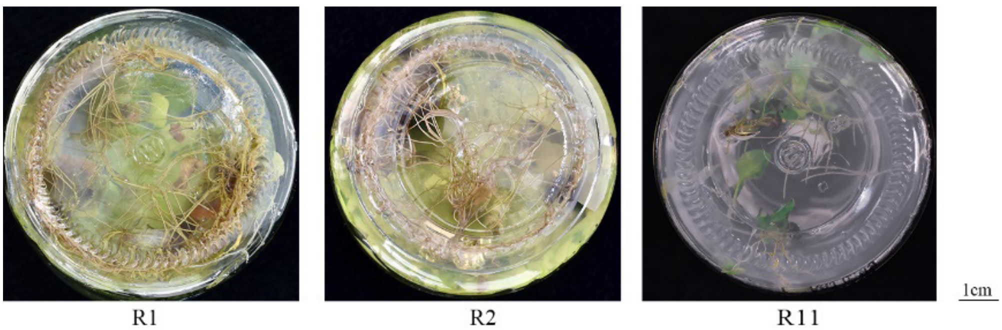

Figure 2. Rooting of 'Msu' in R1, R2, and R11 media.

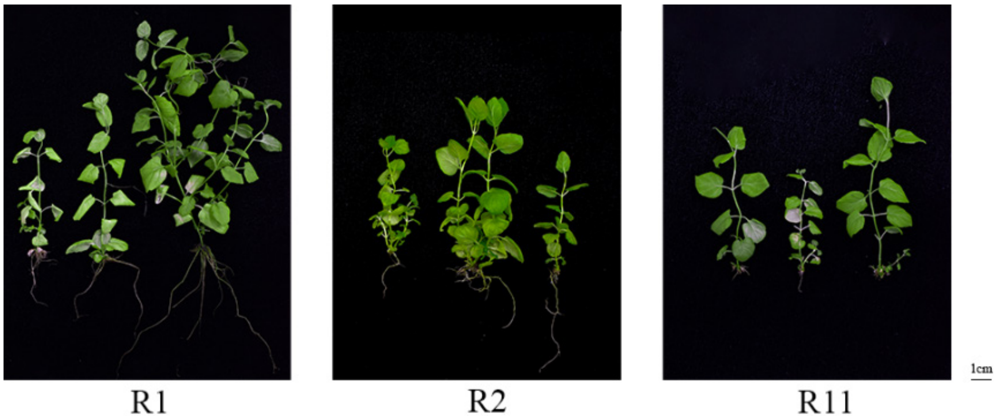

Figure 3. Rooted plantlets of 'Msu' in R1, R2, and R11 media.

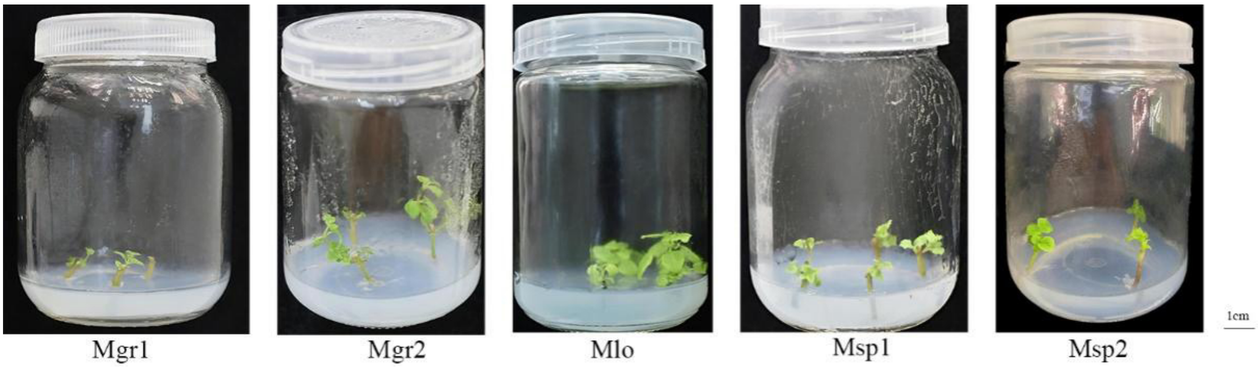

Figure 4. Axillary bud induction of different mint varieties on M1 medium.

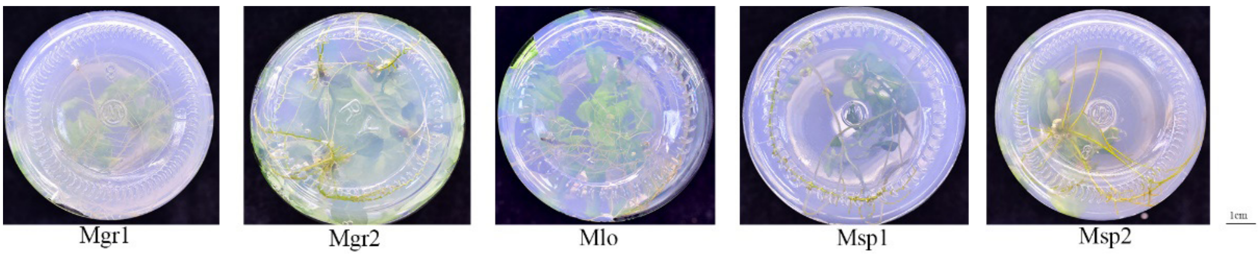

Figure 5. Rooting of different mint varieties on R1 medium.

'Mlo' (domestically collected material), 'Msp1' and 'Msp2' (foreign cultivars). As shown in Table 4, all five mint varieties exhibited axillary bud induction rates exceeding 85% in M1 medium containing 1 mg/L 6-BA + 0.05 mg/L NAA. The regenerated shoots achieved rooting rates ranging from 72.3% to 87.3% in R1 medium supplemented with 0.1 mg/L NAA.

Table 4. Axillary bud induction and rooting rates of different mint varieties in 'Msu'-optimized media.

Variety	Axillary bud induction rate (%)	Rooting rate (%)
'Mgr1'	86.2 ± 2.3cd	75.6 ± 1.3c
'Mgr2'	94.5 ± 1.1a	82.4 ± 1.2b
'Mlo'	89.1 ± 2.1bc	72.3 ± 2.1d
'Msp1'	85.6 ± 1.3d	87.3 ± 1.4a
'Msp2'	91.7 ± 1.2ab	85.8 ± 1.6a

Note: Same letters within a column indicate no significant difference ($P > 0.05$).

Fig. 4 demonstrates significant axillary bud growth in all five mint varieties after 10 days of culture in M1 medium. When shoots reached 2–3 cm in height and were transferred to R1 medium, visible root formation was observed after 14 days of cultivation (Fig. 5). These results indicate that the optimal media combination developed for 'Msu' shows good applicability to other mint varieties. Although it may not represent the absolute optimal conditions for each variety, it effectively induces axillary bud growth and rooting, fulfilling the requirements for tissue culture propagation purposes.

Discussion and conclusion

Multiple factors influence plant tissue culture, including species-specific material characteristics, explant types, and medium composition, all of which significantly affect regeneration efficiency. As a vital aromatic crop and medicinal plant, mint holds substantial market demand. However, the genetic diversity and frequent hybridization among mint germplasms necessitate the use of tissue culture for rapid propagation and germplasm purification. Furthermore, optimizing tissue culture conditions provides a foundation for future genetic transformation and germplasm improvement. Although both stems and leaves of mint can be induced to differentiate, preliminary experiments in this study revealed that stem segments outperformed leaves (which exhibited low callus induction rates and high mortality) in terms of bud induction efficiency, growth speed, and operational tolerance. By selecting nodal stem segments as explants, surface-sterilizing with 2% NaClO for 17 minutes, and utilizing M1 medium (MS + 1 mg/L 6-BA + 0.05 mg/L NAA) for axillary bud induction and R1 medium (1/2 MS + 0.1 mg/L NAA) for root differentiation, regenerated plantlets were obtained within 50–60 days. Experiments on six mint varieties with distinct aromas and genetic backgrounds demonstrated overall regeneration rates ranging from 64% to 100%, indicating the broad applicability of this protocol. These results confirm that tissue culture is a viable propagation method for mint, enabling the production of genetically stable, pathogen-free plants with minimal hormone requirements and short cycles.

The regulatory role of exogenous growth hormones in plant morphogenesis lies in their ability to recalibrate the balance of endogenous auxins and cytokinins (Lu et al. 2022). A defined ratio of cytokinin to auxin typically facilitates the induction of callus, adventitious shoots, or roots, with their relative concentrations governing cell division, redifferentiation, and organogenesis. Previous studies on mint tissue culture predominantly employed combinations of 6-BA with NAA or IAA for shoot induction. For instance, Qian et al. (2008) reported a 70% shoot regeneration rate in *Mentha canadensis* using MS medium supplemented with 1.0 mg/L NAA or 2.0 mg/L IAA. Wu et al. (2018) achieved an 85% bud induction rate in *Mentha spicata* nodal stem segments cultured on MS medium containing 1.0 mg/L 6-BA and 0.2 mg/L NAA. In this study, comparative analysis of 6-BA and NAA ratios revealed that the M1 combination (1.0 mg/L 6-BA + 0.05 mg/L NAA) yielded the most significant results for the hybrid mint 'Msu', not only initiating visible bud growth as early as 4 days of culture but also achieving the highest induction rate. This protocol also demonstrated high efficacy (85.6%–94.5%) across the five additional mint varieties.

Rooting is a critical step in establishing a robust regeneration system, as well-developed roots are essential for the survival of tissue-cultured plantlets during transplantation. Qian et al. (2008) demonstrated that low NAA concentrations combined with 1/2 MS medium effectively promoted root formation. In this study, the R1 treatment (0 mg/L 6-BA + 0.1 mg/L NAA) proved optimal for root induction in 'Msu', producing abundant roots with high efficiency (72.3%–87.3% across varieties). While existing reports on mint tissue culture primarily focus on limited materials such as *Mentha spicata*, this study establishes a simplified and efficient protocol validated across six mint varieties. The methodology offers a scalable approach for rapid germplasm propagation and serves as a valuable reference for future research and industrial applications.

Author contributions

Haowen Zheng: Software, Methodology, Data curation, Writing-Original; Liguo Zhang &: Yexuan Wang: Conceptualization, Methodology, Writing-review & editing; Yang Zhao & Wentao Wang: Software, Methodology, Formal analysis; Yaqiong Qiu: revised the draft to enhance its academic quality.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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