International Journal of Agriculture System
Vol. 13 Issue 2, December 2025
Nationally Accredited Journal Decree No. 177/E/KPT/2024
P-ISSN: 2337-9782, E-ISSN: 2580-6815. **DOI: 10.20956/ijas.v13i2.6762**

Comparison of 2,4-D Alone and 2,4-D–Kinetin Combination on Callus Formation in *Apium graveolens* and *Mentha* sp.

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How to Cite: Jannah, R.M., Firmansah, H. Putri, R.K. (2025). Comparison of 2,4-D Alone and 2,4-D Kinetin Combination on Callus Formation in *Apium graveolens* and *Mentha* sp. *Int. J. Agr. Syst. 13*(2): 152-161

ABSTRACT

Celery (Apium graveolens L.) and mint (Mentha sp.) are economically important aromatic crops. However, their propagation through seeds and vegetative methods is often inefficient due to low seed viability, inconsistent germination, contamination risks, and poor plantlet uniformity. Therefore, this study assessed the response of two plant growth regulator formulations for callus induction in both species under in vitro conditions using a descriptive analysis. Two media treatments were tested: MD1 (Murashige and Skoog (MS) + 1.2 mg L^{-1} 2,4-D) and MD2 (MS + 0.3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin). Observations were conducted on days 7, 14, 21, and 35 after induction (DAI) to assess explant survival, callus formation, and morphology. MD2 medium showed a tendency toward higher explant survival and greater callus formation percentages than MD1 in both species under the observed conditions. Callus initiation occurred between 7 and 14 DAI, with MD2 producing a greater proportion of viable and actively proliferating calli, predominantly characterized by friable-to-compact textures and lighter coloration. In contrast, explants cultured on MD1 exhibited a higher incidence of tissue browning and reduced callus viability, indicating the inhibitory effects associated with high auxin concentrations. These results suggest that a balanced auxin–cytokinin combination provides a more favorable hormonal environment for early callus induction than a single high-dose auxin treatment alone. Therefore, MD2 medium is recommended as an optimal formulation for early-stage callus induction in celery and mint, with potential applications in micropropagation.

Keywords:
Auxins; celery; cytokinins; mint; tissue culture.

1. Introduction

Celery (*Apium graveolens* L.) and mint (*Mentha* sp.) are economically important aromatic herbs widely utilized in the food, pharmaceutical, and cosmetic industries because of their rich profiles of secondary metabolites. Celery contains key bioactive compounds, including flavonoids, phenolics, glycosides, essential oils, and amino acids, such as asparagine and glutamine, which contribute to its antioxidant, anti-inflammatory, and antihypertensive activities (Daoud et al., 2025; Humaira & Amien, 2019; Makarova et al., 2022). Mint, an important source of essential oils, is characterized by high concentrations of menthol, menthone, and pulegone, along with various phenolic compounds that exhibit antimicrobial, antioxidant, and anti-inflammatory properties (Brahmi et al., 2017; Rassem et al., 2018; Salehi et al., 2018). The chemical stability, biological activity, and broad industrial applicability of both species highlight their importance as functional raw materials and their potential for further biotechnological development.

Celery faces several challenges when propagated by seeds or vegetatively. Bruznican et al. reported that celery seeds often exhibit low quality, irregular germination, and high susceptibility to seed-borne pathogens, all of which reduce seed viability and seedling health. Collin and Isaac also stated that callus and shoot regeneration in celery strongly depend on the balance between auxins and cytokinins, while the tissues are highly susceptible to contamination, resulting in low multiplication rates. Mint plants also experience similar challenges. Many mint species are sterile hybrids with very low fertility, making genetic improvement through conventional crossing difficult (Lawrence, 2007). Because mint relies heavily on vegetative propagation, it is more susceptible to carrying soil-borne pathogens and exhibiting somaclonal variation. Consequently, mint plantlets often vary in quality and lack uniformity (Dung, 2020; Duta-Cornescu et al., 2023). These issues indicate that both celery and mint require alternative and more controlled methods for propagation and breeding.

In vitro tissue culture is a practical approach for plant propagation and genetic improvement, enabling the rapid production of pathogen-free and genetically uniform plantlets (Hasnain et al., 2022; Sen et al., 2014). Callus induction is highly dependent on the composition of the culture medium, particularly the type and concentration of plant growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin, which regulate cellular dedifferentiation and proliferation through auxin–cytokinin signaling interactions (Ikeuchi et al., 2013; Mastuti et al., 2017). Recent studies on *Physalis angulata* and *Amorphophallus muelleri* have demonstrated that callogenic responses to 2,4-D and kinetin are highly species-specific, indicating that optimal hormonal requirements cannot be generalized across taxa (Agung et al., 2023; Dwitara et al., 2023).

Despite the economic importance of celery and mint, studies on callus induction in these species remain limited and have primarily focused on regeneration or micropropagation, with little attention given to the comparative effects of single-auxin treatments versus balanced auxin–cytokinin combinations during early callus induction. Moreover, the effects of high auxin concentrations on callus viability and tissue browning in these species have not been systematically evaluated. The concentrations used in this study were selected based on previously reported effective ranges for herbaceous and medicinal plants, in which low to moderate concentrations of 2,4-D (0.2–1.5 mg L^{-1}) promote cellular dedifferentiation, whereas kinetin (0.3–1.0 mg L^{-1}) enhances callus proliferation and quality (Fibriani, 2023; Kakkar et al., 2025; Noli et al., 2024). Therefore, this study aimed to descriptively evaluate the response of celery and mint explants to two plant growth regulator formulations—a single high-dose auxin treatment and a balanced 2,4-D–kinetin combination—under *in vitro* conditions, with particular emphasis on early callus induction and explant survival. This study addresses a methodological gap by directly comparing these two hormonal formulations.

2. Materials and Methods

2.1. Study Site

This study was conducted in Tissue Culture Laboratory 3, Department of Agronomy and Horticulture, IPB University, Bogor, West Java, Indonesia (6°33'36"S, 106°43'34"E). The study was conducted from April to May 2022.

2.2. Plant Materials

Aseptic *in vitro* plantlets of celery (*Apium graveolens* L.) and mint (*Mentha* sp.) were used as the source materials. Nodal explants measuring 0.8–1.0 cm were excised from healthy, actively growing shoots. Culture media were prepared using Murashige and Skoog (MS) basal medium supplemented with sucrose and agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C and 15 psi for 20 min.

The main equipment used in this study included a laminar airflow cabinet, autoclave, analytical balance, pH meter, hot plate magnetic stirrer, culture bottles, scalpels, forceps, measuring cylinders, and growth racks with fluorescent lamps.

2.3. Explant Preparation and Culture Media

Nodal explants (0.8–1.0 cm) were prepared by removing leaf remnants and damaged tissues under aseptic conditions in a laminar airflow cabinet before culture initiation. Each explant was vertically inoculated onto Murashige and Skoog (MS) basal medium supplemented with one of two plant growth regulator formulations: MD1 (MS + 1.2 mg L^{-1} 2,4-D) or MD2 (MS + 0.3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin).

Each treatment was applied to both species with nine replicates per species. Each replication consisted of a single culture bottle containing six explants, resulting in a total of 36 experimental units (culture bottles) and 216 explants.

2.4. Culture Conditions and Observation Parameters

Cultures were maintained for five weeks under controlled growth-room conditions at 20 ± 2 °C with a light intensity of approximately 1000 lux under continuous illumination. Observations were conducted on 7, 14, 21, and 35 days after induction (DAI).

The quantitative parameters included explant survival and callus formation percentages, calculated as the proportion of viable explants or explants forming callus relative to the total number of explants per treatment. Explants were classified as surviving when the tissues remained green or cream-colored, without visible necrosis. Callus formation was recorded when unorganized masses of cells developed at the cut surface or along the explant tissue, following the commonly accepted criteria for callus induction (Normasari et al., 2023).

2.5. Data Analysis and Callus Characterization

Data were analyzed descriptively to identify trends in explant survival and callus induction among treatments, as the study was designed as a preliminary comparative evaluation rather than a hypothesis-testing experiment. This descriptive approach is commonly applied in early-stage callus induction studies aimed at protocol optimization rather than statistical hypothesis testing (Bao et al., 2024).

Qualitative callus characteristics were evaluated by a single trained observer using predefined categorical criteria based on color (white, cream, light brown, or dark brown), texture (friable, compact, or clustered), and relative size (small, medium, or large) to ensure scoring consistency. These morphological classification systems are widely used in *in vitro* callus evaluation to infer physiological status and oxidative responses (Mahood et al., 2026).

3. Results and Discussion

The results indicated that the composition of plant growth regulators in the culture medium strongly influenced explant survival and callus induction in celery (*Apium graveolens* L.) and mint (*Mentha* sp.) (Figure 1). The MD2 medium (MS + 0.3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin) tended to exhibit a higher proportion of viable explants and callus formation than MD1 medium (MS + 1.2 mg L^{-1} 2,4-D) under the observed conditions. This finding suggests that the single auxin concentration tested (1.2 mg L^{-1} 2,4-D) did not enhance callogenic responses under the present experimental conditions and may have been less favourable for cellular proliferation than the balanced auxin–cytokinin treatment, as indicated in previous studies (Liu et al., 2024; Mosoh and Vendrame, 2025). Therefore, the moderate auxin–cytokinin combination in MD2 created a more balanced hormonal environment that supported callus initiation and proliferation than the other treatments.

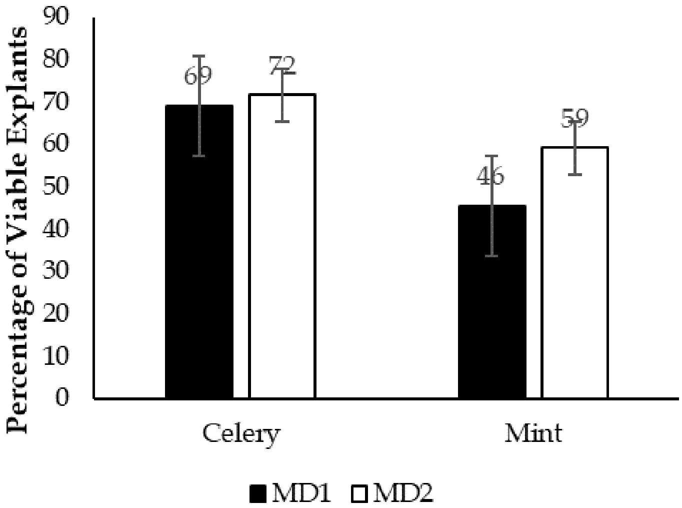

Figure 1. Percentage of viable explants on MD1 and MD2 media

Callus formation that began at 7–14 DAI on both media showed that celery and mint explants responded quickly to changes in the culture environment. Auxin compounds, such as 2,4-D, are known to activate genes related to cell division and dedifferentiation (Lee et al., 2024; Xu et al., 2024), whereas kinetin stimulates mitosis and increases metabolic activity in tissues that support callus formation (Avilez-Montalvo et al., 2022). The characteristics of callus colour and texture observed in celery and mint explants cultured on MD1 and MD2 media are summarized in Table 1. In celery, compact callus was the predominant morphology in both MD1 and MD2 treatments (55.6%), followed

ISSN: 2337-9782, **E-ISSN:** 2580-6815

by friable callus (33.3% in MD1 and 22.2% in MD2). The callus colour was predominantly white in both treatments (88.9%), with only 11.1% showing brown pigmentation. In mint, MD1 primarily produced compact calli (83.3%), with most calli exhibiting brown coloration (83.3%). In contrast, MD2 showed a higher proportion of white callus (77.8%) and a predominance of compact morphology (66.7%), with smaller proportions of friable and clustered callus types. These differences indicate varying levels of cell proliferation, cell wall lignification, and oxidation or phenolic accumulation in the callus tissues. The accumulation of lignin and polyphenols has been reported in previous studies to be associated with callus browning. Thus, the brown to dark brown calli that exhibited reduced viability in the present study may reflect lignification and oxidative processes, although these biochemical parameters were not directly measured (Liu et al., 2024; Wu et al., 2024; Luo et al., 2025).

Table 1. Characteristics of callus colour and texture in celery and mint explants cultured on MD1 and MD2 media

Plant material	Medium	Compact callus (%)	Friable callus (%)	White callus (%)	Brown callus (%)
Celery	MD1	55.6	33.3	88.9	11.1
Celery	MD2	55.6	22.2	88.9	11.1
Mint	MD1	83.3	–	16.7	83.3
Mint	MD2	66.7	11.1	77.8	22.2

Note: MD1 = MS + 1.2 mg L^{-1} 2,4-D; MD2 = MS + 0.3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin.

The composition of the culture medium and auxin–cytokinin ratio directly influenced callus morphology (Figure 2). The type and ratio of plant growth regulators determined whether the callus developed a friable texture (which is easier to suspend and suitable for cell cultures) or a compact texture (denser and often associated with different differentiation potentials) (Bojko et al., 2024; Normasari et al., 2023). Observations of callus morphology, including colour and texture, are commonly used as indirect indicators of physiological conditions during in vitro culture. Colour changes, particularly browning, have been associated in previous studies with oxidative processes, the activity of enzymes such as PPO and POD, and phenylpropanoid pathways leading to lignin formation; however, these parameters were not directly measured in the present study. Increased concentrations of growth regulators, such as 2,4-D and cytokinins, have been reported to influence callus morphology, sometimes resulting in denser and darker tissues that may reflect altered proliferation dynamics and phenolic stress under specific culture conditions (Rybin et al., 2024). In addition, research on *Anredera cordifolia* reported that white-to-brown calli with compact textures were associated with unbalanced exogenous hormone ratios and high oxidation levels (Aulia and Habibah, 2024).

The results demonstrated that an appropriate balance between auxin and cytokinin is a key determinant of successful callus induction, quality, and viability in celery and mint. Previous transcriptomic studies have reported that auxin–cytokinin interactions regulate the transcriptional networks associated with cellular dedifferentiation and proliferation, including genes related to pluripotency, cell cycle progression, and cell wall formation (e.g., LBDs and ARFs) (Duan et al., 2023; Park et al., 2023). In addition, the literature suggests that an imbalanced auxin–cytokinin ratio may disrupt redox homeostasis, potentially leading to increased oxidative stress and the accumulation of reactive oxygen species and phenylpropanoid-related compounds, which have been associated with reduced callus viability and regenerative capacity (Wen et al., 2025; Yue et al., 2024). However, these molecular and biochemical processes were not directly examined in the present study, which evaluated callus responses based on morphological observations under the tested culture conditions.

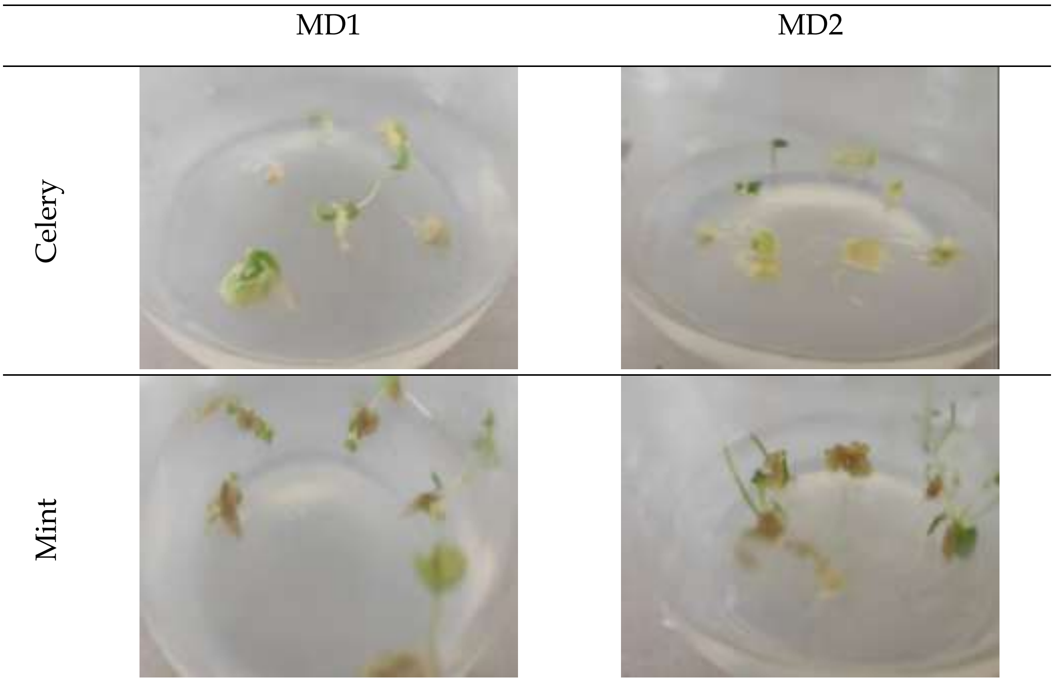

Figure 2. Morphological responses of celery and mint explants on MD1 and MD2 media.

This study showed that the MD2 medium (MS + 0.3 mg L^{-1} 2,4-D + 0.5 mg L^{-1} kinetin) was associated with a more favourable culture response than the single-auxin treatment, as reflected by higher explant survival and more stable callus formation based on morphological observations. These findings are consistent with previous reports on herbal and aromatic plants, which highlight the superiority of balanced auxin–cytokinin combinations over auxin alone for callus proliferation and quality (Xuan et al., 2023). Collectively, this study provides preliminary descriptive information that may support the refinement of in vitro culture protocols for celery and mint, particularly for early callus induction under the tested conditions of this study.

4. Conclusion

This study suggests that the interaction between 2,4-D and kinetin may influence the effectiveness and quality of callus induction in celery (*Apium graveolens* L.) and mint (*Mentha* sp.) under tested conditions. A balanced combination of 2,4-D and kinetin promoted a more stable physiological state that supported cellular dedifferentiation and sustained callus development, in contrast to using a single high concentration of 2,4-D. These findings emphasize the importance of optimizing auxin–cytokinin ratios when designing *in vitro* culture systems for aromatic and medicinal plant propagation. The MD2 medium, which integrates moderate levels of 2,4-D and kinetin, may serve as a preliminary framework for further applications. However, additional validation, including the evaluation of regeneration efficiency and metabolite profiling, is required before broader implementation. This study contributes to the refinement of species-specific tissue culture protocols and supports the development of efficient and reproducible callus-based biotechnological strategies for celery and mint.

Acknowledgements
The authors would like to thank the Tissue Culture Laboratory, Department of Agronomy and Horticulture, IPB University, for providing laboratory facilities and technical support during the implementation of this study. The authors also acknowledge the academic support and constructive discussions provided by the colleagues who contributed to the completion of this study.

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