Micropropagation of Kratom (*Mitragyna speciosa* (Korth.) Havil.): Disinfectants, Growth Regulators, and Low-Cost Chemical Disinfection for *In Vitro* Establishment

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Abstract

Kratom, *Mitragyna speciosa* (Korth.) Havil. is a medicinal plant native to Southeast Asia that is renowned for its therapeutic properties and potential in treating various ailments. Despite its significance, the cultivation and propagation of Kratom have been limited. The purpose of the present study was to develop a cost-effective micropropagation protocol for Kratom by investigating the disinfection efficiency and cost-effectiveness of various chemical disinfectants, optimizing plant growth regulator concentrations, and assessing cost-effective media sterilization methods. The results demonstrated that double disinfection with commercial bleach at 20% and 15% for 5 min each was the most cost-effective treatment for surface disinfection of Kratom seeds, achieving a high disinfection rate (96.67±2.89%) and survival rate (73.33±2.89%) at a relatively low cost (1.46 baht per experiment) compared to mercuric chloride ($HgCl_2$) treatments. The evaluation of benzyladenine (BA) and naphthaleneacetic acid (NAA) effects on *in vitro* growth revealed that the control treatment (MS (Murashige and Skoog medium) without growth regulators) exhibited the best overall growth performance. Among the low-cost disinfectants tested for the culture medium, while autoclaving offers superior disinfection efficacy, commercial bleach at 2 ml/l emerged as the most cost-effective option, especially for resource-limited operations. The final choice, however, should align with specific operational requirements, including scale, resources, and the need for complete disinfection. The successful establishment of a cost-effective micropropagation protocol using low-cost chemical disinfectants and optimized plant growth regulator concentrations can significantly reduce the production costs associated with tissue culture techniques, making the micropropagation of Kratom more economically viable and accessible for large-scale production. The findings from this research provide insights into cost-effective micropropagation methods for Kratom, which will prove valuable for future studies and applications in the field.

Keywords: Kratom, low-cost tissue culture, chemical disinfection, plant growth hormones

Introduction

Kratom, *Mitragyna speciosa* (Korth.) Havil. is a Southeast Asian plant with a rich history of medicinal use dating back to the 19th century (Hassan et al., 2013; Singh et al., 2017). Traditionally, Kratom leaves are believed to treat various ailments and enhance physical strength, making them popular among manual laborers (Nakaphan et al., 2016; Suwanlert, 1975). Recent pharmacological studies have unveiled a diverse array of bioactive compounds in Kratom, with mitragynine as the primary alkaloid and 7-hydroxymitragynine demonstrating superior analgesic properties (Prevete et al., 2023). The pain-relieving effects of mitragynine, mediated through opioid receptors, have been confirmed by both *in vitro* and animal studies (Matsumoto et al., 1996; Todd et al., 2020). Beyond its analgesic properties, Kratom exhibits anti-diarrheal, anti-inflammatory, and anti-depressant effects, and shows promise in addiction treatment, particularly for opioid withdrawal (Saingam et al., 2016; Warner et al., 2016). Despite its therapeutic potential, research on Kratom cultivation and development remained limited until recently, partly due to legal restrictions. In Thailand, Kratom was classified as a narcotic under the 1979 Narcotic Drugs Act until August 24, 2021 (Sengnon et al., 2023).

The Act's revision in 2021 declassified Kratom, paving the way for unrestricted research. Subsequently, the Kratom Plant Act, B.E. 2565 (2022), effective August 27, 2022, now regulates Kratom-related commerce, overseeing its trade, use, research, and product endorsement while acknowledging its economic value (Charoenratana et al., 2021).

Considering these developments, the Thai government is actively promoting Kratom research for medical use, with a focus on cultivation and variety development. However, traditional propagation methods, such as seed dispersal and grafting, face significant challenges in large-scale production and standardization. Seed propagation often results in genetic variability and unpredictable growth patterns, while grafting is labor-intensive and yields limited output. Both methods are susceptible to environmental factors and pest infestations, potentially compromising product quality and consistency (Phromchan, 2023). These limitations underscore the need for more efficient and controlled propagation techniques to meet the growing demand for Kratom in medical research and potential therapeutic applications.

Plant tissue culture techniques offer a promising solution for Kratom propagation, providing an efficient method to generate disease-free and superior planting materials while overcoming the limitations of traditional propagation methods. These techniques rely on proper sterilization methods to eliminate microbial contamination, a critical step in ensuring successful cultures (Webster et al., 2003). The use of plant growth regulators, such as auxins and cytokinins, is crucial in facilitating tissue transformation and organ development, necessitating research to determine optimal concentrations for micropropagation under sterile conditions (Gaba, 2005). Studies on Kratom tissue culture have demonstrated the effectiveness of various growth regulators in inducing desired responses, with thidiazuron (TDZ) enriched woody plant medium (WPM) showing promise in enhancing shoot quantity (Phongprueksapattana et al., 2008). Additionally, research into the effects of different growth regulator concentrations on callus initiation and mitragynine production has provided valuable insights for optimizing cultivation practices, highlighting the potential of tissue culture in advancing Kratom research and production (Mohamad Zuldin et al., 2013).

However, plant tissue culture is often associated with high costs due to the requirement of various chemicals and expensive equipment, such as autoclaves and laminar flow cabinets, which pose significant challenges to the commercialization of tissue culture techniques. Therefore, exploring methods to reduce the cost of plant tissue culture is a topic of interest and holds great importance for promoting the efficient and economically viable propagation of medicinal plants like Kratom. The efficacy of micropropagation is strongly dependent on the efficient disinfection of explants, as the presence of microbial contamination can hinder the growth and maturation of cultured tissues. Various disinfection agents, such as sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$) have been commonly used for surface disinfection of plant explants. However, these disinfectants can be costly and may have negative effects on plant growth and development (Bakhsh et al., 2016). Recent studies have explored cost-effective alternatives to autoclave sterilization in plant tissue culture media preparation. Chotikadachanarong (2014, 2018) demonstrated that common household disinfectants, such as bleach, effectively sterilize media for various plant species, including *Bacopa caroliniana* and *Exacum affine*. These findings suggest that readily available disinfectants could replace traditional autoclaving methods, potentially making plant tissue culture more accessible and economical. Furthermore, Wamaedeesa et al. (2021) demonstrated that both commercial bleach and povidone-iodine were effective in disinfecting MS medium used

for the micropropagation of *Philodendron* "Ruaysap", highlighting the potential of these accessible disinfectants as substitutes for expensive steam disinfection equipment in plant tissue culture protocols.

Therefore, the purpose of the present study was to develop a cost-effective micropropagation protocol for Kratom by investigating the disinfection efficiency and cost-effectiveness of various chemical disinfectants, optimizing plant growth regulator concentrations, and assessing cost-effective media sterilization methods, and evaluating the efficacy of chemical disinfectants for culture media, as well as eliminating the need for expensive equipment such as autoclaves and laminar flow cabinets. The research outcomes present cost-efficient micropropagation strategies for Kratom, potentially serving as a cornerstone for future scientific inquiries and real-world implementations in this field.

Methods and Materials

Disinfection of Kratom Seeds

Kratom seeds were sourced from southern Thailand and were employed as explants for establishing aseptic *in vitro* cultures. The seeds underwent a meticulous cleaning process by initially soaked in clean water, washed with dishwashing liquid, and thoroughly rinsed under running water for 10 min. The fruit was dissected to isolate the seeds. Subsequently, the isolated seeds were immersed in 95% ethanol for 5 min under continuous agitation, followed by draining and air-drying. To evaluate the efficacy of two disinfectants—commercial bleach (Haiter®, Kao Industrial (Thailand) Co., Ltd., containing 6% w/w NaOCl) and $HgCl_2$ (Loba Chemie™, India)—were used in surface disinfection of the Kratom seeds, with thirteen treatments implemented. These treatments varied in disinfectant concentration and exposure duration: single applications of 10%, 15%, and 20% commercial bleach for 5 min; double applications of commercial bleach at 10% and 5%, 15% and 10%, and 20% and 15% for 5 min each; 0.1% $HgCl_2$ for 2, 4, and 8 min; 0.2% $HgCl_2$ for 2, 4, and 8 min; and negative control. Post-treatment, the seeds were subjected to additional cleaning with 1-2 drops of dishwashing liquid, followed by three 5-minute rinses with sterile distilled water. The disinfected seeds were then cultured on MS medium (Murashige & Skoog, 1962), with one seed per tube, and incubated under controlled conditions: fluorescent light (16 hr photoperiod, 2000 lux) at 25 ± 2°C for 4 weeks. Each treatment comprised 20 explants with three replicates. The disinfection rate, survival rate, and associated chemical costs were meticulously recorded and subjected to statistical analysis.

Effects of Plant Growth Regulators

Kratom shoot tip explants (about 1 cm single-node segments) developed from the seeds that had been treated with the most effective surface disinfection protocol (as found in the previous experiment), were selected and grown on MS medium that had different growth regulators added to it. Seven treatments were administered: MS medium without growth regulators; MS + BA at 1, 2, and 4 mg/l; and MS + NAA at 1, 2, and 4 mg/l. Cultures were maintained in a clean environment at 25 ± 2°C under fluorescent light (16 hr photoperiods, 2000 lux) for 8 weeks. Growth parameters, including average leaf number, leaf width, leaf length (largest leaf), shoot height, root number, and root length (longest root), were measured (Figure 1). Each treatment included 10 explants with three replicates, and the results were statistically analyzed.

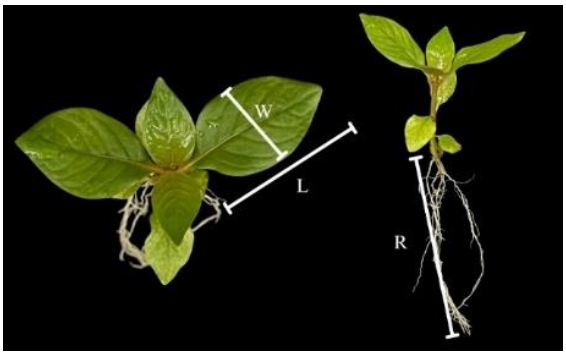

Figure 1 The measurement methods for *M. speciosa* plant: (W) leaf width, (L) leaf length and (R) root length

Plant Tissue Culture Media Disinfection

MS medium was prepared by precise measurement of components, with the volume adjusted using distilled water to achieve a solution containing 30 g/l sucrose. The pH was calibrated to 5.6-5.7, followed by the addition of 7 g/l agar. The medium was then heated until complete agar dissolution and subsequently cooled to 60°C before the introduction of chemical disinfectants. In the tissue culture media disinfection experiment, two disinfecting agents were employed: povidone-iodine solution (Betadine®, containing 10% povidone-iodine) and commercial bleach (Haiter®). Eight disinfection treatments were evaluated: commercial bleach at concentrations of 2, 4, and 6 ml/l; povidone-iodine at 0.5, 1, and 2 ml/l; an autoclaved positive control; and a negative control without disinfection. The prepared media were aliquoted into 4 oz tissue culture bottles, with 20 ml per bottle. Uncontaminated Kratom shoot tips, obtained from seeds subjected to the most effective surface disinfection treatment as identified in the preceding experiments, were sectioned into approximately 1 cm single-node explants and individually cultured on the media (one explant per tube). The cultures were maintained under controlled conditions: fluorescent light (16 hr photoperiod, 2000 lux) at 25 ± 2°C for 8 weeks. Each treatment comprised 10 explants with three replicates. Multiple parameters were assessed and statistically analyzed, including disinfection percentage, survival percentage, average root length, shoot height, leaf number, and the associated costs of chemicals and equipment.

Statistical Analysis

All experiments were conducted using a completely randomized design (CRD). Data collected from the various studies were subjected to statistical analysis, and the significance of differences between means was determined using analysis of variance (ANOVA).

Results

The Effects of Disinfectant Type and Concentration on the Disinfection Efficiency of Kratom Seeds

An evaluation of disinfectant types and concentrations on the disinfection rate of Kratom seeds revealed that a double application of commercial bleach 20% followed by 15% for 5 min each and 0.2% $HgCl_2$ for 8 min exposure both achieved maximum disinfection rates of 96.67±2.89% and 98.33±2.89% ($p \leq 0.05$). However, lower concentrations and shorter exposure times significantly reduced disinfection efficacy. For instance, 0.1% $HgCl_2$ for 2 min exposure resulted in a minimal disinfection rate of 75.00±5.00%, while 10% commercial bleach for 5 min exposure yielded the lowest percentage of disinfected seeds at 48.33±7.64%. To test seed viability, sequential applications of commercial bleach 10% followed by 5%, and 15% followed by 10% for 5

min each produced the highest statistically significant survival rates of 85.00±5.00% ($p \leq 0.05$). Conversely, 0.2% $HgCl_2$ for 8 min exposure resulted in the lowest survival rate of 43.33±7.64% (Table 1).

Cost analysis revealed that treatments utilizing 0.2% $HgCl_2$ for 2, 4, and 8 min exposures were the most expensive at 10.93 baht per experiment. The most economical treatment was 10% commercial bleach for 5 min (0.42 baht per experiment). When assessing overall cost-effectiveness, the double application of commercial bleach 20% followed by 15% for 5 min each emerged as the most effective treatment. This method demonstrated a relatively low cost (1.46 baht per experiment), significantly less than $HgCl_2$ treatments (5.47 to 10.93 baht per experiment). This method also achieved a high disinfection rate (96.67±2.89%) which was comparable to the most effective treatments, while maintaining a superior survival rate (73.33±2.89%). Consequently, based on the combined factors of cost, disinfection efficiency, and survival rate, this treatment proved to be the most cost-effective option for Kratom seed disinfection (Table 1).

Table 1 Cost of chemical disinfectant, percentage of disinfection, and survival after 4 weeks using commercial bleach and $HgCl_2$ for surface sterilization of *M. speciosa* seeds

Treatments				% Disinfection (Mean±SD)	% Survival (Mean±SD)	Cost of chemical disinfectant (Baht/experiment)
1st Surface disinfection	Time (min)	2nd Surface disinfection	Time (min)			
10% commercial bleach	5	-	-	48.33±7.64 h	68.33±2.89 cd	0.42
15% commercial bleach	5	-	-	65.00±5.00 g	71.67±5.77 bcd	0.63
20% commercial bleach	5	-	-	68.33±2.89 fg	81.67±2.89 ab	0.83
10% commercial bleach	5	5% commercial bleach	5	83.33±5.77 cd	85.00±5.00 a	0.63
15% commercial bleach	5	10% commercial bleach	5	88.33±2.89 bc	85.00±5.00 a	1.04
20% commercial bleach	5	15% commercial bleach	5	96.67±2.89 a	73.33±2.89 bcd	1.46
0.1% $HgCl_2$	2	-	-	75.00±5.00 ef	76.67±2.89 abc	5.47
0.1% $HgCl_2$	4	-	-	88.33±2.89 bc	63.33±7.64 de	5.47
0.1% $HgCl_2$	8	-	-	91.67±2.89 ab	50.00±5.00 fg	5.47
0.2% $HgCl_2$	2	-	-	78.33±5.77 de	68.33±2.89 cd	10.93
0.2% $HgCl_2$	4	-	-	83.33±2.89 cd	55.00±13.23 ef	10.93
0.2% $HgCl_2$	8	-	-	98.33±2.89 a	43.33±7.64 h	10.93
negative control	-	-	-	0.00±0.00 i	20.00±5.00i	0
F-test				*	*	
CV%				33.1	27.9	

Values in the same column with different letters are statistically significant at a significance level of $p \leq 0.05$, as determined by the Duncan Multiple Range Test. Commercial bleach, (Haiter®, 600 ml) costs 25 baht, while $HgCl_2$ (100 g, Loba Chemie™, India) costs 5,465 baht. One experiment refers to the preparation of 1 L of MS medium.

Effects of BA and NAA Concentrations in MS Medium on *In Vitro* Growth of Kratom

Explants for this experiment were obtained from the most effective disinfection treatment: a double disinfection protocol using commercial bleach at 20% followed by 15%, each applied for 5 min, which yielded the highest disinfection. Among BA treatments, 1 mg/l BA produced the highest average leaf number (10.84±0.15), significantly outperforming higher concentrations of 2 mg/l (8.98±0.20) and 4 mg/l (8.27±0.13). However, this improvement in leaf production was offset by reductions in other growth parameters compared to the control. Notably, 4 mg/l BA substantially restricted growth across all measured parameters, indicating potential toxicity at higher concentrations. For NAA treatments, 1 mg/l NAA yielded the widest

leaves (1.54±0.04 cm) compared to 2 mg/l (1.04±0.14 cm) and 4 mg/l (0.91±0.33 cm). However, NAA negatively impacted other growth metrics, even at the lowest concentration. Higher NAA concentrations progressively inhibited overall plant development, with 4 mg/l NAA showing the most severe growth restrictions in terms of leaf number, leaf length, shoot height, root number, and root length. Comparing the efficacy of BA and NAA, neither consistently promoted comprehensive growth improvement. Both plant growth regulators enhanced specific aspects of morphology at low concentrations (1 mg/l) but generally restricted growth across multiple parameters, especially at higher doses (2 mg/l and 4 mg/l). This suggests that while these regulators can influence certain growth characteristics, they may not be suitable for the overall healthy development of Kratom *in vitro*.

Notably, the control treatment (MS medium without growth regulators) demonstrated superior performance across all measured growth parameters. It produced the highest average leaf number (10.09±0.07), leaf length (2.39±0.07 cm), shoot height (3.40±0.03 cm), root number (3.24±0.14), and root length (2.25±0.21 cm). This comprehensive growth superiority in the absence of additional plant growth regulators is a significant finding. Therefore, the untreated MS medium emerged as the most effective treatment for promoting the overall healthy growth of Kratom *in vitro*. While low concentrations of BA and NAA showed some beneficial effects on specific growth parameters, these improvements were outweighed by growth restrictions in other areas. These findings suggest that maintaining Kratom cultures under maximum control conditions (MS medium without growth regulators) would be most beneficial for future experiments and potential large-scale propagation efforts (Table 2 and Figure 2).

Table 2 Effects of BA and NAA concentrations in MS medium on average leaf number, leaf width, leaf length, shoot height, root number, and root length *in vitro* growth of *M. speciosa*

Treatment	Average leaf no.	Average leaf width (cm)	Average leaf length (cm)	Average shoot length (cm)	Average root no.	Average root length (cm)
MS	10.09±0.07 b	1.46±0.13 ab	2.39±0.07 a	3.40±0.03 a	3.24±0.14 a	2.25±0.21 a
MS+1 mg/l BA	10.84±0.15 a	1.47±0.18 ab	2.15±0.03 b	3.18±0.02 b	2.58±0.20 b	0.70±0.07 c
MS+2 mg/l BA	8.98±0.20 c	1.15±0.06 bc	1.37±0.01 c	2.41±0.07 d	1.80±0.18 c	1.10±0.15 b
MS+4 mg/l BA	8.27±0.13 d	0.88±0.26 c	1.05±0.06 e	2.18±0.07 e	1.71±0.33 c	0.61±0.05 c
MS+1 mg/l NAA	9.91±0.07 b	1.54±0.04 a	2.1±0.02 b	2.71±0.13 c	2.84±0.28 ab	0.92±0.04 b
MS+2 mg/l NAA	8.22±0.08 d	1.04±0.14 c	1.17±0.04 d	2.33±0.07 d	1.98±0.39 c	1.04±0.08 b
MS+4 mg/l NAA	7.64±0.07 e	0.91±0.33 c	0.95±0.02 f	2.08±0.10 e	1.67±0.18 c	0.53±0.04 c
F-test	*	*	*	*	*	*
CV%	12.7	25.3	44.2	21.4	28.9	63.8

Values in the same column with different letters are statistically significant at a significance level of $p \leq 0.05$, as determined by the Duncan Multiple Range Test.

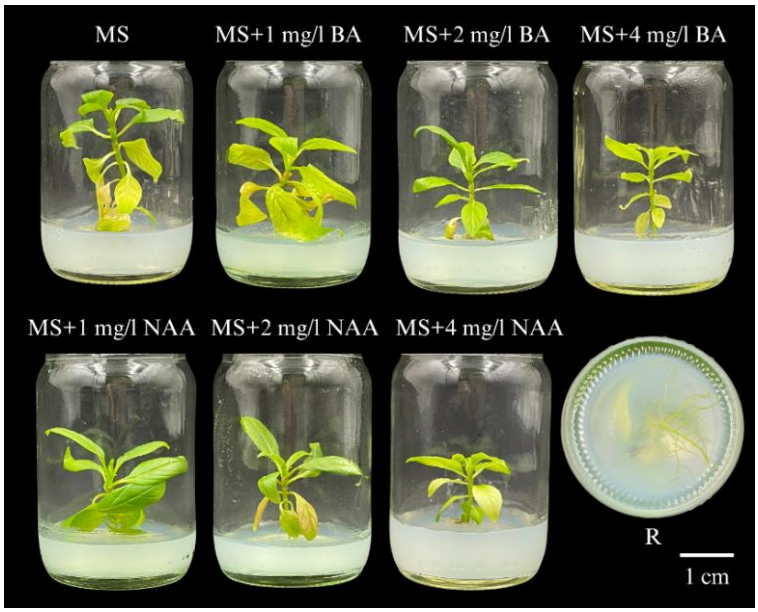

Figure 2 Micropropagation of *M. speciosa* explant on different BA and NAA concentrations in MS medium: MS, MS+1 mg/l BA, MS+2 mg/l BA, MS+4 mg/l BA, MS+1 mg/l NAA, MS+2 mg/l NAA, MS+4 mg/l NAA, R represent root formation in MS medium

Effects of Commercial Bleach and Povidone-Iodine Solution Disinfectant Concentrations in MS Medium on *In Vitro* Growth of Kratom

Analogous to the plant growth regulators experiment, explants were derived from the double disinfection protocol (20% then 15% commercial bleach, 5 min each), which maximized disinfection. Among commercial bleach treatments, 2 ml/l emerged as the most effective concentration, balancing disinfection efficiency (50.00±10.00%) with a high survival rate (66.67±5.77%). This concentration also promoted superior growth, yielding the highest average root length (1.98±0.02 cm), shoot length (2.83±0.19 cm), and leaf number (10.36±0.43). Higher concentrations of 4 ml/l and 6 ml/l improved disinfection rates but led to decreased survival and growth parameters. For povidone-iodine solutions, 0.5 ml/l demonstrated the best overall performance. It achieved a good balance between disinfection rate (73.33±5.77%) and survival rate (76.67±5.77%), while maintaining moderate growth parameters. Higher concentrations (1 ml/l and 2 ml/l) exhibited increased disinfection efficiency; however, this was offset by reduced survival rates and significantly inhibited growth.

Comparing the two disinfectants, commercial bleach at 2 ml/l outperformed povidone-iodine in promoting robust growth while maintaining effective disinfection. Although 0.5 ml/l povidone-iodine showed a slightly higher disinfection rate (73.33±5.77% vs 50.00±10.00%) and survival rate (76.67±5.77% vs 66.67±5.77%), it resulted in inferior growth metrics compared to 2 ml/l commercial bleach, particularly in root length (0.89±0.14 cm vs 1.98±0.02 cm), shoot length (1.22±0.13 cm vs 2.83±0.19 cm), and leaf number (8.17±0.35 vs 10.36±0.43). Therefore, 2 ml/l commercial bleach emerged as the most favorable treatment, offering the best balance between disinfection efficacy, survival rate, and growth promotion. This concentration is recommended for future *in vitro* Kratom cultivation experiments, as it consistently demonstrated superior performance across all evaluated parameters (Table 3 and Figure 3).

Table 3 Percentage of disinfection, survival rate, root length, shoot length, and leaf number of *M. speciosa* explants after 8 weeks cultivated in MS medium with varying doses of commercial bleach and povidone-iodine solution

Treatment	% Disinfection (Mean±SD)	% Survival (Mean±SD)	Average root length (cm)	Average shoot length (cm)	Average leaf no.
commercial bleach 2 ml/l	50.00±10.00 d	66.67±5.77 bc	1.98±0.02 a	2.83±0.19 a	10.36±0.43 a
commercial bleach 4 ml/l	70.00±10.00 c	66.67±5.77 bc	1.48±0.07 c	1.93±0.13 b	8.94±0.19 b
commercial bleach 6 ml/l	96.67±5.77 a	46.67±5.77 d	1.29±0.19 d	1.63±0.08 c	8.56±0.33 bc
povidone-iodine 0.5 ml/l	73.33±5.77 bc	76.67±5.77 b	0.89±0.14 e	1.22±0.13 d	8.17±0.35 cd
povidone-iodine 1 ml/l	76.67±5.77 bc	66.67±5.77 bc	0.67±0.06 f	1.22±0.18 d	8.03±0.38 d
povidone-iodine 2 ml/l	86.67±11.55 ab	56.67±11.55 cd	0.54±0.04 f	0.84±0.07 e	2.99±0.22 e
positive control	100.00±0.00 a	93.33±5.77 a	1.78±0.02 b	2.95±0.06 a	10.03±0.17 a
negative control	0.00±0.00 e	0.00±0.00 e	0.00±0.00 g	0.00±0.00 f	0.00±0.00 f
F-test	*	*	*	*	*
CV%	41.50	41.50	62.70	65.70	44.50

Values in the same column with different letters are statistically significant at a significance level of $p \leq 0.05$, as determined by the Duncan Multiple Range Test.

Figure 3 Micropropagation of *M. speciosa* explant on different commercial bleach and povidone-iodine solution concentrations in MS medium: commercial bleach 2, 4 and 6 ml/l, povidone-iodine solution 0.5, 1 and 2 ml/l, positive control, negative control, R- bleach indicate root formation in MS medium with commercial bleach and R- povidone-iodine solution indicate root formation in MS medium with povidone-iodine solution

A comparative analysis of commercial bleach, povidone- iodine, and autoclaving for Kratom micropropagation revealed significant differences in cost-effectiveness and disinfection efficacy. Autoclaving demonstrated superior disinfection (100.00±0.00%) and survival rates (93.33±5.77%), but at a substantially higher cost (60,124 baht). In contrast, 2 ml/l commercial bleach, while showing lower disinfection efficiency (50.00±10.00%), offered comparable growth parameters and survival rates (66.67±5.77%) at a fraction of the cost (2,604.08 baht). Povidone-iodine solutions, particularly at 0.5 ml/l, showed intermediate performance but at slightly higher costs than commercial bleach (Tables 3 and 4). The cost analysis shown in Table 4

delineates both variable and fixed costs. Variable costs, including culture medium and electricity, differ minimally between chemical treatments but increase significantly for autoclaving due to higher energy consumption. Fixed costs, primarily equipment-related, are substantially higher for autoclaving (60,000 baht) compared to chemical treatments (2,500 baht). This cost structure suggests that chemical disinfection methods may be more economically viable, especially for smaller-scale or resource-limited operations. However, it's crucial to note that chemical disinfection methods do not achieve 100% disinfection, unlike autoclaving. This imperfect disinfection leads to the potential loss of plant material and increased operational costs due to contamination. The 50% disinfection rate of 2 ml/l commercial bleach suggests the potential loss of half the cultures, effectively doubling the cost per successful culture. This factor is crucial when assessing the true cost-effectiveness of chemical disinfection methods. While 2 ml/l commercial bleach appears most cost-effective initially, long-term assessments must consider potential contamination losses. The choice between chemical disinfection and autoclaving should depend on operation scale, available resources, and the need for absolute disinfection in specific applications. This balanced approach ensures the optimal selection of disinfection methods for various research or commercial scenarios.

Table 4 Cost comparison of commercial bleach and povidone-iodine solution disinfectants for *in vitro* growth of *M. speciosa* in MS medium

	List	Cost/Treatment (baht)							
		commercial bleach			povidone-iodine solution			positive control	negative control
		2ml/l	4ml/l	6ml/l	0.5ml/l	1ml/l	2ml/l		
Variable cost	**Culture medium**								
	Medium	100	100	100	100	100	100	100	100
	bleach, Haiter®	0.08	0.16	0.25	-	-	-	-	-
	povidone-iodine, Betadine®	-	-	-	2.17	4.33	8.67	-	-
	Total cost of culture medium	100.08	100.16	100.25	102.17	104.33	108.67	100	100
	Electricity cost								
	Autoclave	-	-	-	-	-	-	24	-
	Induction cooker	4	4	4	4	4	4	-	4
Total variable cost		104.08	104.16	104.25	106.17	108.33	112.67	124	104
Fixed cost	**Equipment**								
	Autoclave	-	-	-	-	-	-	60,000	-
	Pot	500	500	500	500	500	500	-	500
	Induction cooker	2,000	2,000	2,000	2,000	2,000	2,000	-	2,000
Total fixed cost		2,500.00	2,500.00	2,500.00	2,500.00	2,500.00	2,500.00	60,000.00	2,500.00
Total cost		2,604.08	2,604.16	2,604.25	2,606.17	2,608.33	2,612.67	60,124.00	2,604.00

The disinfectants include commercial bleach, Haiter®, (600 ml, 25 baht) and povidone-iodine solution, Betadine® (15 ml, 65 baht). Equipment used was a 50 L autoclave unit (3,000 watts) (LS-50LDitoplanding™), 2.5 L pots (ZEBRA®), and an induction cooker (2,000 watts) (Electrolux®). The electricity cost is calculated using the formula: (power (watts) x usage time (hours) x electricity rate) / 1000, with the sterilization times being 2 hours for the autoclave and 0.5 hours for the boiling pot. The electricity rate is 4 baht per unit (as of 2023). One experiment refers to the preparation of 1 L of MS medium.

Discussion

Efficacy of Chemical Disinfectants in Disinfecting Kratom Seeds

Commercial bleach (NaOCl) and mercuric chloride ($HgCl_2$) are effective disinfectants in plant tissue culture, each with distinct mechanisms. NaOCl produces hypochlorous acid (HOCl) in solution, penetrating cell walls and disrupting protein synthesis, while $HgCl_2$ binds to protein sulfhydryl groups, inactivating enzymes and disrupting cell membranes (Fukuzaki, 2006; Urbano et al., 2024). These mechanisms explain their efficacy across various plant species. Previous studies have demonstrated the significant impact of disinfectant type, concentration, and exposure time on both disinfection efficacy and explant survival across various plant species. In *Rhododendron wardii* seeds, 15% NaOCl with Tween 20 for 20 min achieved 100% disinfection but only 51% germination, while lower concentrations increased germination but compromised disinfection (Amarasinghe et al., 2018). For *Aquilaria malaccensis*, 0.1% $HgCl_2$ for 15-30 sec effectively disinfected explants, while 0.2% for 12 min maximized seed viability (Nurul et al., 2012). In *Staphylea bumalda* DC., 1% $HgCl_2$ with 70% alcohol for 5 min effectively disinfected shoot tips (Xiulei et al., 2012). A study on *Cyathea latebrosa* showed both 0.1% $HgCl_2$ and 30% NaOCl were effective in spore disinfection (Nadhirah et al., 2022). These studies underscore the critical role of disinfection parameters in achieving successful outcomes while maintaining explant viability. The variability in effective protocols across species emphasizes the need for careful optimization. Despite $HgCl_2$'s effectiveness, NaOCl is often preferred due to its lower toxicity and high efficacy (Babu et al., 2022), highlighting the importance of safety in disinfectant selection for plant tissue culture. Our research on Kratom aligns with and extends these findings, as well as studies on *Dioscorea* spp. (Sookruksawong, 2022, 2023) and *Mammillaria plumosa* (Sookruksawong, 2024). The Kratom seed disinfection protocol used higher NaOCl concentrations (1.2% and 0.9%, from 20% and 15% bleach) with shorter exposure times (5 min each), achieving 96.67±2.89% disinfection and 73.33±2.89% survival. This contrasts with Sookruksawong's (2022) work on *D. bulbifera*, where lower NaOCl concentrations (0.9% and 0.6%) but longer exposure times (15 and 10 min) achieved 100% disinfection and survival. These results highlight the interplay between disinfectant concentration and exposure time. Generally, higher concentrations and longer exposure times increase disinfection rates but may reduce survival rates. Notably, lower concentrations with extended exposure times can achieve high disinfection rates by allowing prolonged contact between the disinfectant and explant surface. This underscores the importance of exposure time as a critical factor in disinfection protocols. The varying effectiveness of these disinfectants across species and explant types emphasizes the need for tailored protocols in different tissue culture applications. Weber et al. (2015) further demonstrated NaOCl's cost-effectiveness in potato micropropagation. Collectively, these findings suggest that commercial bleach provides a balanced combination of safety, efficacy, and adaptability for various plant tissue culture applications, including Kratom micropropagation.

Effects of BA and NAA on the *In Vitro* Growth of Kratom

Benzyladenine (BA) and naphthaleneacetic acid (NAA) are crucial plant growth regulators in tissue culture. BA influences growth, metabolism, and rejuvenation processes, while NAA affects root formation, hormone levels, and overall plant development (Abas et al., 2021; Liu et al., 2018). The concentrations of BA and NAA significantly impact plant growth in tissue culture, with effective levels varying across species, influencing shoot and root development, leaf formation, and overall vigor. For rambutan (*Nephelium lappaceum* L.), a

combination of 0.5 ppm NAA and 4 ppm BA significantly enhanced shoot and root development in lateral shoot explants *in vitro* (Yuniastuti et al., 2022). In pineapples (*Ananas comosus* (L.) Merr.), shoot growth was promoted with 1-3 mg/l NAA or 1-5 mg/l BA, with a combination of 1 mg/l NAA and 4 mg/l BA enhancing shoot formation and proliferation (Zulkarnain & Neliyati, 2017). These studies underscore the importance of precise BA and NAA concentrations in maximizing species-specific plant growth in tissue culture. In the present study, 1 mg/l was found to be the most effective concentration for both BA in leaf production and NAA in leaf width expansion. Higher concentrations (2-4 mg/l) of either hormone inhibited overall growth. These findings align with previous studies on various medicinal plants (Jader & Obaid, 2023; Wijaya et al., 2022). However, high concentrations of BA and NAA can adversely affect plant growth and development. The study on rambutan lateral shoot explants indicates that while certain concentrations of BA and NAA can promote growth, the balance is crucial; excessive amounts can lead to suboptimal outcomes (Yuniastuti et al., 2022). This suggests that excessive BA and NAA can disrupt the natural hormonal balance required for root and shoot development. Interestingly, the control treatment containing only MS medium elicited the greatest growth across all measured parameters. This indicates that the endogenous levels of plant growth regulators in Kratom explants may be sufficient to support maximum growth under the tested conditions. This aligns with the understanding that while growth regulators like BA and NAA are essential for tissue culture growth, their efficacy is highly concentration-dependent. Higher concentrations can lead to poor growth and development by disrupting physiological and biochemical processes essential for plant growth (Katarzyna & Przemysław, 2010; Zulkarnain & Neliyati, 2017).

The Effectiveness of Commercial Bleach and Povidone-Iodine Solution in Disinfecting MS Medium for Kratom

Chemical alternatives to thermal disinfection in plant tissue culture have gained attention for their cost-effectiveness and efficiency. Sodium hypochlorite (NaOCl) and povidone-iodine have emerged as promising disinfectants, effectively reducing microbial contamination while minimizing harm to plant tissues. Pais et al. (2016) demonstrated that 0.003% active chlorine from NaOCl effectively controlled contamination in Gerbera hybrida cv. Essandre micropropagation, yielding growth comparable to thermal disinfection. Thepsithar and Thongpukdee (2013) found that 0.36% (v/v) povidone-iodine in *Phalaenopsis* culture media achieved 100% disinfection and supported protocorm growth equivalent to autoclaved media. Povidone-iodine's broad-spectrum antimicrobial properties have been noted across various fields (Alsaedy et al., 2023; Nair et al., 2023). Additionally, Thepsithar et al. (2013) reported that combining povidone-iodine with essential oils enhanced shoot explant growth. However, the efficacy of these disinfectants is concentration-dependent. Our study on Kratom revealed an inverse relationship between disinfectant concentration and plant viability. For NaOCl, 6 ml/l (0.036%) achieved maximum disinfection (96.67±5.77%) but lowest survival (46.67±5.77%), while 2 ml/l (0.012%) provided the best balance (50.00±10.00% disinfection, 66.67±5.77% survival). For povidone-iodine, 0.5 ml/l offered the most favorable outcome (73.33±5.77% disinfection, 76.67±5.77% survival). Interestingly, Sookruksawong (2022) reported 100% disinfection for *D. bulbifera* cultures across all NaOCl concentrations in MS medium, contrasting with our findings for Kratom where disinfection rates ranged from 50.00±10.00% to 96.67±5.77%. This discrepancy may be attributed to several factors, primarily the difference in experimental durations: 4 weeks for *D. bulbifera* versus 8 weeks for Kratom. Remarkably, extended culture periods without subculturing can lead to NaOCl concentration reduction, attributed to atmospheric CO_2

interaction, thermal and photolytic degradation, pH-induced instability, and volatile chlorine gas formation (Fukuzaki, 2006). These processes diminish NaOCl's disinfection potency over time, potentially facilitating microbial growth. While chemical disinfection methods offer potential benefits, especially for large-scale or resource-limited operations, their effectiveness varies with plant species and culture conditions. Further research is needed to establish broadly applicable protocols, considering the dynamic nature of chemical disinfectants in extended tissue culture, particularly for slow-growing species like Kratom.

Our cost analysis of disinfection methods in Kratom micropropagation revealed significant economic differences between chemical disinfectants and autoclaving. Chemical methods, particularly commercial bleach and povidone-iodine demonstrated lower total costs due to substantially reduced fixed costs (2,500 baht vs 60,000 baht for autoclaving) and slightly lower variable costs. The results of this study are consistent with recent research conducted by Da Costa Urtiga et al. (2019) and Duan et al. (2019), which found that chemical disinfectants can effectively be substituted for autoclaving in different plant species without negatively affecting their growth. However, Pais et al. (2016) emphasized the importance of optimizing disinfectant concentration and exposure time to balance efficacy and plant viability. While autoclaving showed higher disinfection efficiency and survival rates, its significantly higher total cost (60,124 baht) made it less economically viable for Kratom micropropagation, echoing observations by Simran Chandrahas & Narasimhan (2021) on autoclaving's high operational costs. The choice between chemical disinfection and autoclaving should be based on the scale of operation, available resources, and the critical need for absolute sterility in specific research or commercial applications. For Kratom micropropagation, our results suggest that chemical disinfection offers a cost-effective approach, balancing efficacy, plant viability, and economic considerations.

Conclusion and Suggestions

In conclusion, this study provides valuable insights into Kratom micropropagation. Commercial bleach (NaOCl) effectively disinfected Kratom seeds, with a double disinfection protocol (20% followed by 15%, 5 min each) achieving high disinfection and survival rates. For *in vitro* growth, BA and NAA at 1 mg/l showed some positive effects, but MS medium without growth regulators produced the best overall growth. While autoclaving offers superior disinfection efficacy, commercial bleach at 2 ml/l (0.012% NaOCl) emerges as the most cost-effective option for disinfecting MS medium, balancing disinfection, survival, and economics. This method proves particularly advantageous for resource-limited and large-scale operations. However, the final selection should align with specific operational requirements, including scale, resources, and the need for complete disinfection. Future research should focus on fine-tuning disinfectant concentrations and exposure times for various Kratom genotypes, exploring lower concentrations and combinations of growth regulators, and investigating the long-term effects of chemical disinfectants on media composition and plant growth. These findings contribute to developing improved protocols for Kratom tissue culture, potentially facilitating its conservation and sustainable utilization.

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Author 1 (Suchonma Sookruksawong): The author solely conducted all aspects of this research, including conceptualization, methodology, data collection, analysis, and manuscript preparation.

Conflict of Interests

The author declares no conflicts of interest.

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