RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2025.016

ScienceAsia 51 (1): 2025: ID 2025016: **1–9**

In vitro seed germination and phytochemical content of kaffir lime (*Citrus hystrix* DC.) in response to auxin and cytokinin treatments

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Received 10 Oct 2023, Accepted 16 Jan 2025
Available online 17 Mar 2025

ABSTRACT: Kaffir lime leaf extract has a cytotoxic effect on cancer cells. This study aimed to increase the availability of aseptic and controlled leaves using *in vitro* seed germination to enhance the production of secondary metabolites. The growth of *in vitro* plantlets is influenced by plant growth regulators (PGRs) such as auxin and cytokinins, with auxin promoting rooting and cytokinins inducing leaf growth. The objective was to determine the optimal use of PGRs for kaffir lime seed germination and analyze the phytochemical content in the leaves. Kaffir lime seeds were germinated on Murashige and Skoog (MS) medium without PGRs (MS0). On day 21, the seedlings were treated with 1-naphthaleneacetic acid (NAA) as the auxin and thidiazuron (TDZ), 6-benzylaminopurine (BAP), and kinetin (KIN) as the cytokinins. Seedling growth was assessed by measuring root length, plantlet height, leaf width and height, and the number of leaves. On day 35, the leaves were harvested and analyzed using gas chromatography-mass spectrometry. The results showed that the seeds underwent germination on day 3. Adding 0.2 mg/l NAA induced the longest roots in the kaffir lime plantlets. The NAA:TDZ (0.2:1 mg/l) treatment resulted in the tallest plantlets with the longest leaves, whereas the NAA:KIN (0.2:1 mg/l) treatment produced the largest number of leaves. The bioactive compounds detected in the plantlet leaves included cyclotrisiloxane hexamethyl, which was found in all treatments except NAA:BAP (0.2:0.5 mg/l). Terpenoid compounds such as citronellyl isobutyrate were identified in BAP-treated plantlets, while phytol was detected in those grown under MS0 conditions.

KEYWORDS: *in vitro* germination, auxin, cytokinin, growth, bioactive compounds

INTRODUCTION

Previous studies have reported that the bioactive compounds in a kaffir lime leaf extract have antioxidant, anti-inflammatory, antifungal, antimicrobial, antitumor, and analgesic effects [1]. Our previous study revealed that chloroform and ethyl acetate leaf extracts of kaffir lime (*Citrus hystrix* DC.) have a cytotoxic effect on neuroblastoma cancer [2]. However, there are several disadvantages to using kaffir lime leaves as a therapeutic agent, including overexploitation and unstable production of bioactive compounds due to environmental factors.

In vitro seed germination allows more consistent production of aseptic leaves. Sterile plantlets resulting from *in vitro* seed germination can be directly used as raw material for natural medicines as an explant source for callus induction or acclimatized and transferred to soil. The seeds of some citrus species, such as *Citrus aurantium* L., *Citrus sinensis* (L.), *Citrus limon* (L.), and *Citrus reticulata* Blanco, germinate in 5–7 weeks, and the percentage of germination ranges from 19 to 90% in *ex vitro* medium [3]. Therefore, *in vitro* germination is a promising strategy to quickly produce higher percentages of germinating seeds.

Plant growth regulators (PGRs) have been added to culture media to maximize germination and growth of seedlings, particularly auxin and cytokinin hormones. Exogenous auxins such as 1-naphthaleneacetic-acid (NAA) induce rooting and shoots, the development of the meristem, inhibit senescence, and induce fruit development [4]. Cytokinins induce shoot development and stem cells to generate leaf primordial [5]. Cytokinins also stimulate cell division, increase cell expansion, retard senescence, and release plants from dormancy.

Various types of cytokinins were used in this study. Thidiazuron (TDZ) reportedly increases the number of leaves in *Lallemantia iberica* [6]. Benzylaminopurine (BAP) produces the largest number of leaves in *C. sinensis* [7] and *C. hystrix* [8], induces shoot growth in *C. nobilis* from cotyledon explants [9]. Moreover, kinetin produces the greatest number of shoot growth in *C. reticulata* [10].

PGRs also enhance the synthesis of phytochemical compounds to stimulate plant growth. Adding NAA to tissue culture medium prepared for *Balanites aegyptica* increases the production of diosgenin [11]. Another study showed that cytokinins stimulate the production of ajmalicine, scopolamine, scopoletin, and carotenoids. TDZ also increases phenolic and flavonoid compounds in *L. iberica* [6]. BAP enhances phenolic contents in *Stevia rebaudiana* [12]. Supplementing cactus with kinetin during germination increases the production of kaempferol and flavonoid 2 times higher than that in *ex vitro* cacti [13]. Combina-

tions of auxins and cytokinins stimulate the production of secondary metabolites [14].

This study used *in vitro* seed germination as a strategy to produce controlled sterile plantlets as a ready-to-use explant source with consistent production of phytochemical compounds. Therefore, the objective of this study was to determine the optimal PGR concentrations for the growth of kaffir lime seeds and the phytochemical content in the leaves of plantlets.

MATERIALS AND METHODS

Sample preparation

Kaffir lime fruits were collected from Pekutan, Bayan, Purworejo, Central Java, Indonesia. Uniform and spotless 5–6 cm diameter fruits were selected fresh, and none were rotten or moldy. The seeds were hard and homogeneous in size.

Seed induction

The seeds were shelled to remove the outer layer and accelerate water imbibition. Then, the seeds were sterilized in 2 stages with 5.2% sodium hypochlorite (NaClO) for 5 min and 70% alcohol for 5 s, followed by rinsing with sterile distilled water. The seed surface was aseptically incised and cultured on Murashige and Skoog medium (PhytoTech Labs, USA) without PGRs (MS0). The cultures were incubated in the dark for 7 days and then transferred to a light condition. Darkness stimulates the emergence of the radicles, whereas light induces the opening of the cotyledons, the formation of chlorophyll, and prevents etiolation. The plantlets were grown on MS0 medium until day 14.

Subculture and medium transfer

The plantlets were subcultured on day 21 from MS0 medium to MS0 as the control group or transferred from MS0 to MS medium containing NAA or a combination of NAA and various concentrations of cytokinins as the treatment groups. The 0.2 mg/l NAA concentration was obtained from our preliminary study (data not shown). The auxin NAA was sourced from Merck (Germany). The concentrations of the cytokinins were based on several studies. We used 0.5 mg/l [15], 1 mg/l [6], and 3 mg/l [16] of TDZ. We used 0.5 mg/l [7], 1 mg/l [9], and 3 mg/l [8] of BAP, and we used 0.5 mg/l [17], 1 mg/l [18], and 3 mg/l [8] of kinetin. The BAP and kinetin were sourced from Phytotech, while TDZ was obtained from Duchefa Biochemie (Netherlands).

The plantlets were cultured until day 35 with growth parameters measured every 7 days. The measured parameters included root length, plantlet height, leaf width, leaf length, and the number of leaves. Root length was measured as the area below the hypocotyl. Plantlet height was measured as the area between the hypocotyl and the epicotyl. Leaf width was obtained from the widest part of the leaf, while leaf length was measured from the longest part of the leaf.

Bioactive compound analysis of kaffir lime leaf plantlets by gas chromatography-mass spectrometry (GC-MS)

Leaves were harvested on day 35 and dried in an oven until they reached a constant weight. The dried leaves were crushed into a powder and extracted using a maceration method with ethyl acetate as the solvent. The extract was analyzed by GC-MS on an AGILENT 7890A with an MS AGILENT 5977B GC/MS detector (Agilent Technologies, Palo Alto, CA, USA). The MS column was a DB-5MS (5%-*phenyl*)-*methylpolysiloxane*). The NIST 16 library database was used.

Data Analysis

Growth data were analyzed using one-way ANOVA and principal component analysis (PCA). ANOVA was conducted with the DMRT test at a significance level of $p < 0.05$ using SPSS version 25. PCA was carried out with multivariate testing in Minitab version 21 to evaluate the effect of cytokinin on Kaffir lime plantlet growth. Growth parameters were standardized using standard deviation. Principal components were identified based on a cumulative variance $> 70\%$ or an eigenvalue > 1.

RESULTS

Growth response of the seeds

All of the kaffir lime seeds germinated easily in MS0 (100% germination rate), demonstrating that kaffir lime seeds readily germinated without the need for exogenous PGRs to break dormancy. Representative *in vitro* seed germination and seedling growth are presented in Fig. 1(a). The radicles emerged on day 3, a sign that the germination process had finished, and 100% germination was achieved on day 7. The cotyledons emerged on day 10, followed by a true leaf on day 13.

The kaffir lime plantlets were transferred to a new medium on day 21 to study morphology. The plantlets were subcultured on MS0 as a control group and on various concentrations of PGRs as the treatment groups. Fig. 1(b–d) shows that adding NAA and the various cytokinins increased plantlet growth. Fig. 1(b–d) also shows the complete plant parts for all treatments. All treatments — whether MS0, NAA alone, or a combination of NAA with cytokinin — produced plantlets with robust stems and green leaves, indicating healthy growth conditions. Significant differences were observed in parameters such as root length, plantlet height, leaf length, and leaf count compared to the control, while leaf width showed no significant difference. These quantitative differences will be further detailed in Fig. 2 and Table 1.

Fig. 2 shows that the plantlets transferred to medium containing various cytokinins had shorter

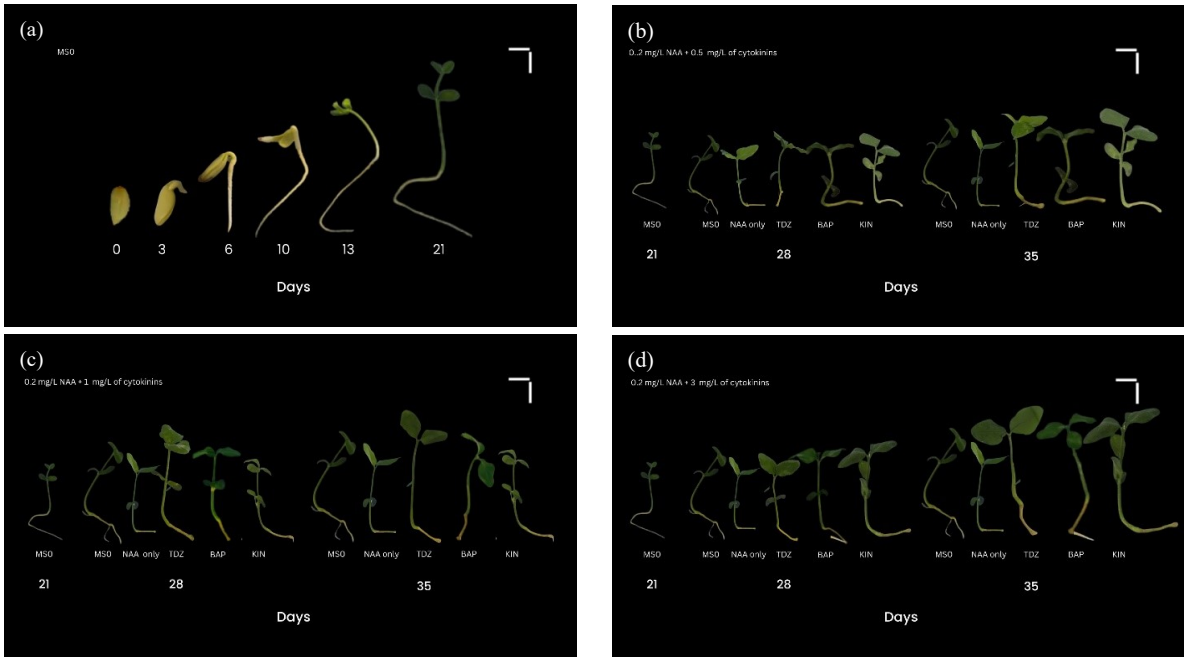

Fig. 1 (a) Representative images of *in vitro* seed germination and seedling growth on MS0 medium. Seedling morphology with the addition of auxin (0.2 mg/l NAA) and various types of cytokinins: (b) 0.5 mg/l cytokinin; (c) 1 mg/l cytokinin; and (d) 3 mg/l cytokinin. Scale bars = 10 mm.

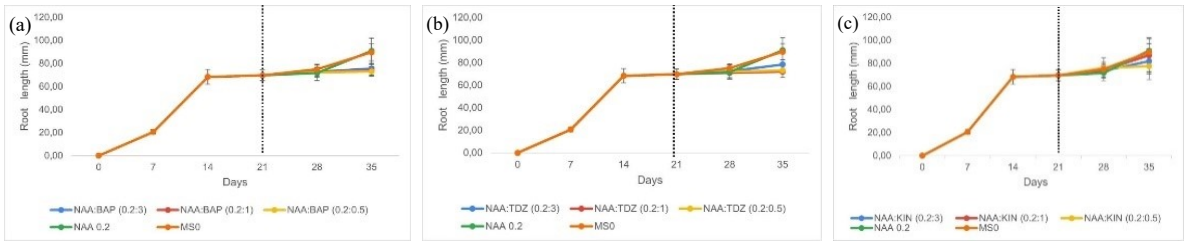

Fig. 2 Root length: (a) Benzylaminopurine (BAP), (b) Thidiazuron (TDZ), and (c) Kinetin (KIN) treatments. (Note: dotted line indicates the time when PGRs were added.)

Table 1 Growth parameters on harvesting day (D35).

Treatment	Growth parameter				
(mg/l)	Root length (mm)	Plantlet height (mm)	Leaf length (mm)	Leaf width (mm)	Leaf number
MS0	89.67 ± 7.23^{b}	44.00 ± 3.60^{cd}	21.67 ± 1.52^{ghi}	14.00 ± 1.00^{kl}	2.00 ± 0.00^{n}
NAA 0.2	91.00 ± 11.00^{b}	43.33 ± 2.51^{c}	20.00 ± 2.00^{g}	13.00 ± 0.00^{k}	2.00 ± 0.00^{n}
NAA:BAP (0.2:0.5)	73.00 ± 4.00^{a}	47.67 ± 1.52^{cdef}	20.67 ± 0.58^{gh}	14.67 ± 0.58^{kl}	2.00 ± 0.00^{n}
NAA:BAP (0.2:1)	73.67 ± 3.78^{a}	51.00 ± 3.60^{ef}	23.33 ± 0.58^{ij}	16.00 ± 1.00^{kl}	2.00 ± 0.00^{n}
NAA:BAP (0.2:3)	75.67 ± 3.78^{ab}	48.33 ± 1.52^{cdef}	22.67 ± 0.58^{hij}	15.50 ± 0.58^{lm}	2.67 ± 0.58^{no}
NAA:TDZ (0.2:0.5)	73.00 ± 2.65^{a}	52.33 ± 2.52^{ef}	21.67 ± 0.58^{ghi}	14.33 ± 0.58^{kl}	2.67 ± 0.58^{n}
NAA:TDZ (0.2:1)	72.00 ± 3.46^{a}	53.00 ± 5.20^{ef}	24.00 ± 1.73^{j}	16.33 ± 1.15^{l}	2.33 ± 0.58^{n}
NAA:TDZ (0.2:3)	78.33 ± 4.04^{ab}	47.00 ± 3.46^{cde}	21.33 ± 0.58^{ghi}	16.00 ± 1.00^{kl}	2.33 ± 0.58^{n}
NAA:KIN (0.2:0.5)	77.67 ± 11.54^{ab}	54.30 ± 2.30^{f}	19.67 ± 1.52^{g}	15.60 ± 2.88^{kl}	3.00 ± 0.00^{op}
NAA:KIN (0.2:1)	87.00 ± 14.17^{ab}	50.30 ± 7.50^{def}	21.30 ± 0.58^{g}	15.30 ± 0.58^{kl}	3.67 ± 0.00^{p}
NAA:KIN (0.2:3)	82.00 ± 10.81^{ab}	50.67 ± 1.15^{def}	21.30 ± 1.15^{ghi}	15.30 ± 0.58^{kl}	2.30 ± 0.00^{n}

Analysis was carried out per column on the growth parameters of each plantlet. Numbers with different letters indicate significant differences based on one-way ANOVA at a significance level of 5%.

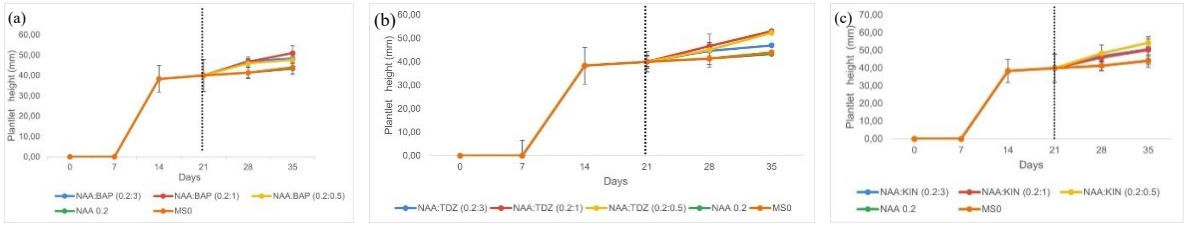

Fig. 3 Plantlet height: (a) BAP, (b) TDZ, and (c) KIN treatments. (Note: dotted line indicates the time when the PGRs were added.)

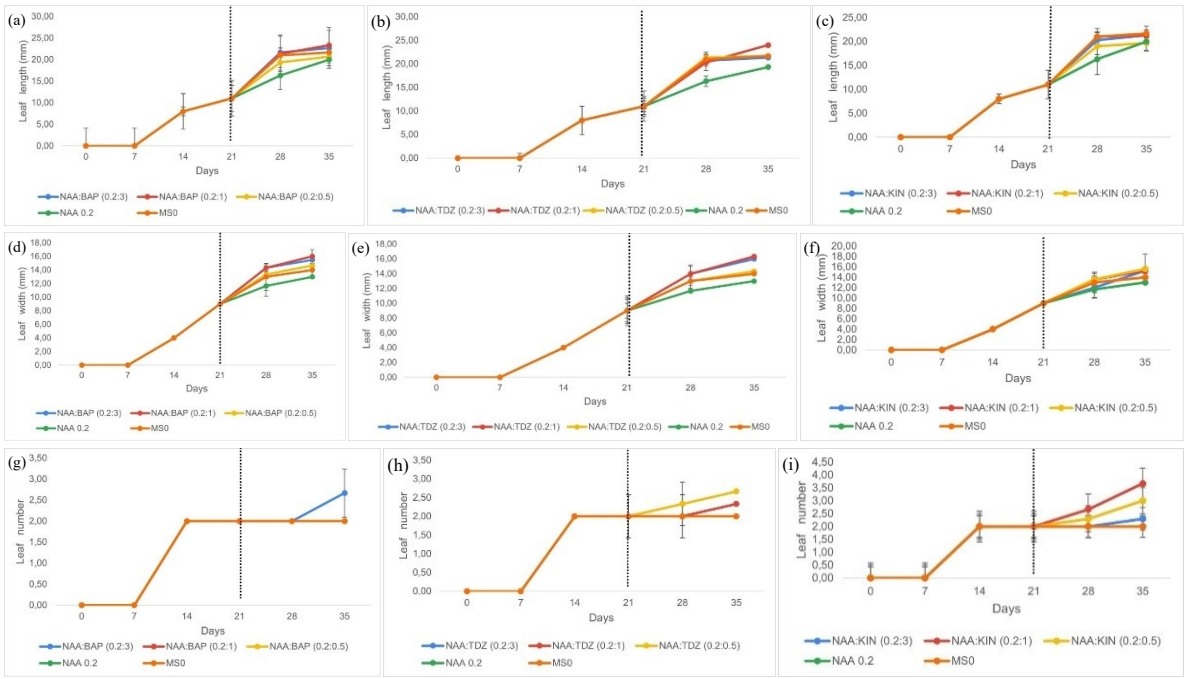

Fig. 4 Leaf length: (a) BAP, (b) TDZ, and (c) KIN treatments; leaf width: (d) BAP, (e) TDZ, and (f) KIN treatments; leaf number of kaffir lime plantlet: (g) BAP, (h) TDZ, and (i) KIN treatments. (Note: dotted line indicates the time when the PGRs were added.)

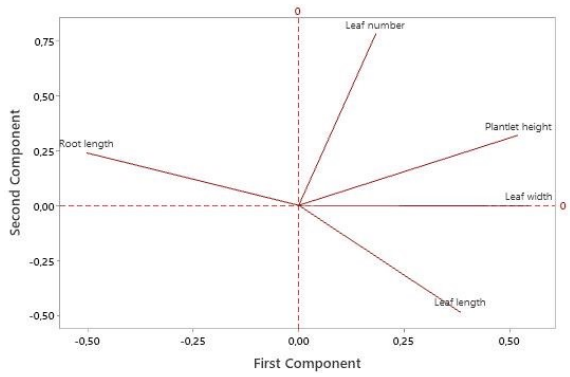

Fig. 5 Principal component analysis (PCA) of different growth parameters following the addition of cytokinin.

roots than those in the 0.2 mg/l NAA and MS0 treatments. The longest roots were observed in 0.2 mg/l NAA medium, while the shortest roots were found in the NAA:TDZ (0.2:1 mg/l) medium.

Fig. 3 shows that the medium containing various cytokines resulted in taller plantlets than those in the 0.2 mg/l NAA and MS0 treatments. The tallest plantlets were found in the NAA:KIN medium (0.2:0.5 mg/l) treatment, while the shortest plantlets were observed in the 0.2 mg/l NAA treatment. Some groups showing significantly taller plantlets than control were NAA:BAP (0.2:1 mg/l), NAA:TDZ (0.2:0.5 and 1 mg/l), and NAA:KIN (0.2:0.5 mg/l).

Fig. 4(a–f) shows that the plantlets in medium containing various cytokinins had longer and wider leaves than those in the 0.2 mg/l NAA and MS0 treatments. The longest and widest leaves were found significantly in the NAA:TDZ (0.2:1 mg/l) treatment, while the shortest and narrowest leaves were in the 0.2 mg/l NAA treatment.

The largest number of leaves (3–4 leaves) was found in plantlets exposed to the NAA:KIN medium (0.2:1 mg/l). The other treatment groups generally

had 2 leaves (Fig. 4(g–i)).

Table 1 shows that the medium containing NAA:TDZ (0.2:1 mg/l) produced the longest leaves, which were significantly different from those in the MS0 treatment. The NAA:KIN medium (0.2:1 mg/l) produced the largest number of leaves, which was significantly different from those in the MS0 treatment. Among all of the treatments, only the leaf width of the plantlets in the PGR treatments was not significantly different from that in the MS0 treatment. Fig. 5 further confirms that cytokinin addition is positively correlated with the number of leaves, plantlet height, leaf width, and leaf length in the first principal component (PC1) but negatively correlated with root length. In the second principal component (PC2), cytokinin addition shows a positive correlation with the number of leaves, plantlet height, leaf width, and root length.

Bioactive compounds

Composition in the culture medium can influence the production of bioactive compounds. Table 2 and Fig. S1 show the compounds present in all treatments. The addition of PGRs was found to decrease the number of bioactive compounds in plantlets compared to MS0. The high-est number of bioactive compounds was observed in MS0, followed by the addition of cytokinin at 1 mg/l BAP and 3 mg/l Kinetin, with 7, 6, and 6 compounds, respectively.

Table 2 shows that cyclotrisiloxane hexamethyl was present in all treatments except NAA:BAP (0.2:0.5 mg/l), with the highest percentage increase observed in the NAA:TDZ (0.2:0.5 mg/l) treatment compared to MS0. Additionally, pentanoic acid and bicyclo[4.1.0]heptane, 7-methylene were detected only in the treatments with PGRs and were absent in the control group. Terpenoid compounds were also identified with phytol found in MS0 and citronellyl isobutyrate in the NAA:BAP (0.2:1 mg/l) treatment. Compounds detected in the MS0, NAA (0.2 mg/l), and cytokinin treatments exhibited antibacterial, antioxidant, anti-inflammatory, and anticancer properties, as summarized in Table 3.

DISCUSSION

In this study, we germinated kaffir lime seeds *in vitro* to produce plantlets with the primary aim of utilizing their leaves as a source of raw material for traditional medicine. The leaves are known for their high concentrations of naturally synthesized bioactive compounds. The novelty of this research lies in the application of various cytokinin treatments to specifically induce kaffir lime leaf growth and optimize leaf production. The leaves generated from these cytokinin-induced plantlets were subsequently used for the extraction of bioactive compounds. Additionally, each organ of the plantlet was evaluated as a potential explant source for callus induction with the possibility of acclimatizing the plantlets for successful transfer to soil.

In citrus species, such as *C. aurantium* L. seedlings), *C. sinensis* (L.) Osbeck cv. Biondo commune, *C. sinensis* (L.) Osbeck seedlings, *C. limon* (L.) Osbeck cv. Femminello, and *C. reticulata* Blanco cv. Tardivo di Ciaculli took 5 to more than 7 weeks to germinate with percentages of germination ranging from 19 to 90% in *ex vitro* medium [3]. Our data indicate more uniform emergence of the radicle under *in vitro* than *ex vitro* conditions (unpublished data). Humidity and water availability are key factors in the germination process. *In vitro* germination provides a more controlled environment, ensuring stable water content and humidity, which accelerates imbibition and promotes germination. Conversely, *ex vitro* conditions are less regulated with fluctuating humidity and water availability, necessitating constant monitoring and water supply. Therefore, *in vitro* germination proves to be more efficient and reliable than *ex vitro* methods.

This study revealed that kaffir lime seeds germinated easily under *in vitro* conditions. The radicle was detected on day 3, and 100% germination was achieved on day 7 after placing the seeds on MS0 medium (Fig. 1(a)). This result was faster than *C. sinensis* (L.) Osbeck cv. Hamlin, which was planted on the same medium. *C. sinensis* (L.) Osbeck cv. Hamlin begins to germinate after 5–9 days, and 50% seed germination is achieved on day 10 [19]. Furthermore, it has been reported that the germination of rough lemon seeds (*C. jambhiri*), Cleopatra mandarin (*C. reshni* Hort ex Tan), carrizo citrante (*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf, and sour orange (*C. aurantium* L.) requires 6.42, 6.87, 11.58, and 13.29 days, respectively, on MS medium + 1 mg/l NAA + 0.5 mg/l BAP [20]. These results can be affected by the type of seed coat, which affects imbibition and germination. The thick tissue of seed coat is difficult for water to enter the embryo. In our study, the kaffir lime (*C. hystrix* DC.) seeds had soft seed coats that were easy to remove, which supported the germination process.

The germination process initiates with seed imbibition or water absorption and concludes with the emergence of the radicle. In this study, the outer seed coat was removed to accelerate the imbibition. Imbibition is critical for triggering cellular responses, as it enhances metabolic activity. Water uptake stimulates the embryo to synthesize gibberellins, which initiate the germination process. Gibberellins also induce the production of α-amylase and β-amylase enzymes, which hydrolyze stored reserves in the endosperm to support seedling growth. Additionally, germination stimulates the synthesis of various endogenous hormones, including auxins, cytokinins, abscisic acid, salicylic acid, ethylene, jasmonic acid, and brassinosteroids [21].

Table 2 A comparison of the phytochemical compound contents between treatments.

No	Compound	Molecular formula	Molecular weight (g/mol)	Group of compound	Peak area (%) MS0	NAA 0.2	NAA 0.2:BAP (mg/l) 0.5	1	3	NAA 0.2:TDZ (mg/l) 0.5	1	3	NAA 0.2:KIN (mg/l) 0.5	1	3
1	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	Fatty acid	16.2	-	-	-	-	-	-	-	-	-	-
2	Phytol	$C_{20}H_{40}O$	296.50	Diterpenoid	5.5	-	-	-	-	-	-	-	-	-	-
3	Bicyclo[10.1.0]tridec-1-ene	$C_{13}H_{22}$	178.31	Fatty acid	52.1	-	-	-	-	-	-	-	-	-	-
4	4-Methyl-2,4-bis(p-hydro-cyphenyl)pent-1-ene	$C_{18}H_{20}O_2$	268.35	Phenol	1.1	8.3	1.8	-	-	32.5	-	9.2	1.8	-	-
5	Cyclotrisiloxane hex -amethyl	$C_6H_{18}O_3Si_3$	222.46	Fatty acid	5.9	28.3	-	13.8	15.9	30.8	22.2	11.5	26.9	13.6	12.8
6	Pentanoic acid	$C_5H_{10}O_2$	102.13	Fatty acid	-	8.1	-	-	-	-	-	-	-	-	-
7	Bicyclo[4.1.0]heptane, 7-methylene	C_8H_{12}	108.18	Fatty acid	-	27.0	-	-	-	-	-	-	-	-	17.6
8	Citronellyl isobutyrate	$C_{14}H_{26}O_2$	226.36	Monoterpenoid	-	-	-	30.4	-	-	-	-	-	-	-

Table 3 Biomedical activity of chemical compounds in the plantlets.

No	Phytochemical compound	Biological activity AC	AO	AI	AM/AB
1.	n-Hexadecanoic acid (Palmitic acid)	[32]			
2.	Phytol		[33]	[33]	
3.	Bicyclo[10.1.0] tridec-1-ene		[34]		[35]
4.	4-Methyl-2,4-bis(p-hydrocyphenyl) pent-1-ene	[36]			
5.	Cyclotrisiloxane hexamethyl		[37]		
6.	Pentanoic acid	[38]			
7.	Bicyclo[4.1.0] heptane, 7-methylene				[39]
8.	Citronellyl isobutyrate	[40]			

AC: Anticancer; AO: Antioxidant; AI: Anti-inflammantory; AM/AB: Antimicrobial/Antibacteria.

After subculturing, the plantlets in MS0 medium, NAA alone, and a combination of NAA and cytokinin exhibited healthy growth. The plantlets had robust stems and green leaves (Table S1). Different PGRs enhance plant growth and the synthesis of bioactive compounds differently. Therefore, it is necessary to determine the best PGR for kaffir lime plantlets. Plantlets in the 0.2 mg/l NAA treatment had the longest roots compared to those in the MS0 and the NAA and cytokinin groups (Fig. 2 and Table 1). NAA loosens the cell wall bonds so that root cells elongate and fill with water. The cell continues to grow and synthesize cell wall materials and cytoplasm. This mechanism results in elongated roots. NAA induces rooting. Each species and explant source has a different NAA concentration requirement. MS + 0.4 mg/l NAA is the best medium to support root growth in *Broussonetia papyrifera* shoot culture [22].

NAA plays a vital role in controlling plant growth by promoting the proliferation, expansion, and elongation of roots. NAA causes recipient stem cells to release H+ toward the primary cell wall. Wall-loosening proteins become active in the acidic environment and enlarge the cell walls. The plant cell expands due to water entering through osmosis. In addition, the inclusion of the proper concentration of cytokinin helps stimulate root elongation and increase the height of the plant, and adding NAA alone does not stimulate rooting or induce the callus. Plantlets in all of the cytokinin treatments had the longest and widest leaves compared to those in the MS0 and 0.2 mg/l NAA treatment groups (Fig. 3, Fig. 4(a–f), Fig. 5, and Table 1). Cytokinins play a role in the differentiation of shoot meristems, and adding cytokinins to the medium initiates the formation of leaves. Cytokinins promote the development of chloroplasts, and leaf shoots support the ability of the plantlets to photosynthesize. The increase in leaf length was due to rapid cell division that encourages cell differentiation. Furthermore, leaf width is closely related to the direction of division, enlargement, and the number and distribution of cells.

Plantlets in most of the cytokinin treatments had more leaves than those in the MS0 and NAA alone groups. The number of new leaves increased after exposure to the combination of NAA + cytokinins (Fig. 4(g–i), Fig. 5, and Table 1). These results indicate that cytokinins play a role in triggering cell division and regulating the number of leaf primordia. Kinetin is a cytokinin PGR that regulates the growth of roots and leaves. In this study, adding kinetin enhanced the formation of leaves compared to the MS0 and the other PGRs. The greatest number of leaves

was detected on the NAA:KIN medium (0.2:1 mg/l). This result agrees with results reported, that adding 0.5 mg/l of each kinetin and NAA produces the largest leaves on buds produced from node explants of *C. aurantifolia* (Christm.) Swing [23]. Accordingly, adding kinetin also resulted in the largest dry and fresh weights of the leaves, which supported our result.

In terms of bioactive compounds in this study, the number of peaks in the medium with added PGRs was less than that in MS0. This phenomenon may have occurred because the PGR exposure was too short (14 days). In addition, the plantlets were too young. Our plantlets were differentiating and forming new organs such as leaves, branches, and roots. In this stage, PGRs increased the formation of plant organs rather than biosynthesizing compounds.

However, prolonged application of exogenous PGRs such as cytokinins can negatively impact plantlet growth by disrupting hormonal homeostasis. Cytokinin exposure beyond 14 days has been observed to induce senescence, marked by leaf abscission (unpublished data, our preliminary study). This prolonged cytokinin exposure is a major factor in promoting leaf senescence. Zdarska et al [24] demonstrated that extended BAP treatment activates abscisic acid-responsive genes, resulting in leaf drop. Similarly, Guo et al [25] reported that the optimal exposure duration for TDZ in *Morus indica* is 8 to 10 days. Consequently, limiting plantlet exposure to exogenous PGRs to less than 14 days is recommended for promoting growth. Furthermore, the synthesis of bioactive compounds can be enhanced by callogenesis and elicitation.

Cyclotrisiloxane hexamethyl was a bioactive fatty acid compound detected in every treatment with a large peak area (Table 2). However, cyclotrisiloxane hexamethyl was not detected in the NAA:BAP (0.2:0.5 mg/l) treatment, likely due to the suboptimal BAP concentration for its fatty acid synthesis. The concentration of BAP and the specific type of fatty acid synthesized significantly influence the fatty acid composition. When BAP levels are suboptimal, fatty acid production may decrease [26]. This is supported by Vetchinnikova et al [26], who found that BAP concentrations of 0.25 mg/l and 0.5 mg/l resulted in the lowest linoleic acid content in the phospholipid fraction.

Cytokinins increase the production of fatty acids in soybeans [27]. Fatty acids are vital components of all organisms and play an important role in plant defense, including crosstalk with hormones and other mediators of plant defense signaling pathways during biotic and abiotic stress [28]. This explains why we found a large number of fatty acid compounds in our study such as n-hexadecanoic acid, bicyclo[10.1.0] tridec-1-ene, cyclotrisiloxane hexamethyl, and bicyclo[4.1.0] heptane, 7-methylene. Every treatment group had the presence of cyclotrisiloxane hexamethyl.

In our study, terpenoid compounds such as phytol were detected in the control, and citronellyl isobutyrate was detected in the NAA:BAP (0.2:1 mg/l) treatment (Table 2). Phytol is a diterpene and a component of chlorophyll. Large amounts of chlorophyll are produced during plant growth and are degraded as a result of aging or stress. Stress changes the ultrastructure of the chloroplast. As a result, the lipid composition changes, the fatty acid ester phytol accumulates, and free phytol is released. Phytol was also identified in our previous study, specifically in kaffir lime cell suspension culture extract [29].

Citronellyl isobutyrate, a monoterpene aldehyde, was detected in our study. This finding is consistent with previous studies on *Hyptis marrubiodes* in that the addition of BAP to *in vitro* cultures stimulated monoterpene production [30]. BAP regulates the HMG-CoA reductase (HMGR) enzyme, which is crucial in the mevalonate pathway. HMGR converts mevalonate into isopentenyl pyrophosphate (IPP), which is isomerized to dimethylallyl pyrophosphate (DMAPP). DMAPP reacts with IPP to form geranyl pyrophosphate (GPP), a key precursor for monoterpenoid biosynthesis. Therefore, BAP plays an essential role in regulating monoterpenoid synthesis. This was further supported by studies on *Cymbopogon citratus*, which reported that BAP treatment induced the production of monoterpenoids, particularly citral, by influencing HMGR activity [31].

The compounds detected in MS0, NAA only, and NAA + cytokinin treatments had antibacterial, antioxidant, anti-inflammatory, and anticancer activities (Table 3). Therefore, plantlets can be used as a valuable source of natural medicine. Furthermore, the synthesis of bioactive compounds can be enhanced by callogenesis and elicitation.

CONCLUSION

Adding NAA and various cytokinins to the medium significantly increased *in vitro* seedling growth parameters such as root length, plant height, leaf length, and the number of leaves. The greatest number of leaves was found in the NAA:KIN (0.2:1 mg/l) treatment. Cyclotrisiloxane hexamethyl was a bioactive compound found in all treatments except NAA:BAP (0.2:0.5 mg/l). Terpenoid compounds and citronellyl isobutyrate were detected in the 1 mg/l BAP treatment, whereas phytol was observed in the MS0 group. In future research, kaffir lime plantlets with the addition of PGRs can be used as a source of sterile explants for the production of secondary metabolite compounds.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.016.

Acknowledgements: This work was supported by Final Project Recognition Grant (RTA program) Universitas Gadjah Mada Number 5075/UN1.P.II/Dit-Lit/PT.01.01/2023 (to W.A.S.T). This study was a compilation of some students' undergraduate theses with additional variables and synthesis.

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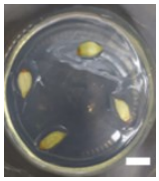
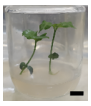
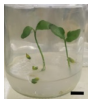
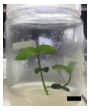
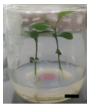
Appendix A. Supplementary data

Table S1 Growth of kaffir lime plantlets under various *in vitro* culture treatments.

MS0 Before treatment						
Day 0	Day 3	Day 6	Day 10	Day 13	Day 21	
						
After treatment (mg/l)						
Day	MS0	NAA (0.2)	NAA:BAP (0.2:0.5)	NAA:BAP (0.2:1)	NAA:BAP (0.2:3)	NAA:TDZ (0.2:0.5)
28						
35						

Day	NAA:TDZ (0.2:1)	NAA:TDZ (0.2:3)	NAA:KIN (0.2:0.5)	NAA:KIN (0.2:1)	NAA:KIN (0.2:3)
28					
35					

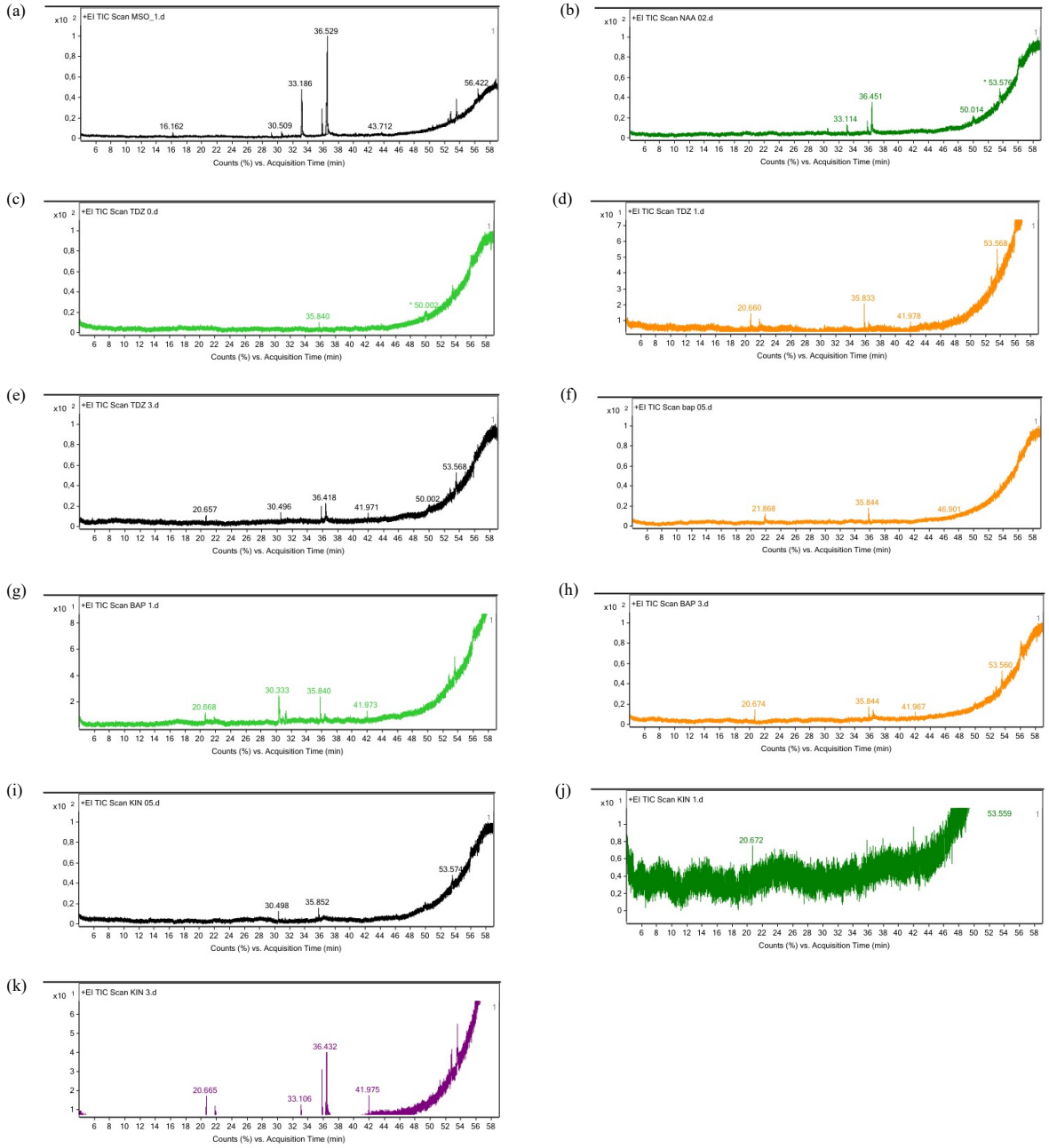


Fig. S1 Gas Chromatography-Mass Spectroscopy (GC-MS) chromatograms. (a) MS0; (b) NAA 0.2 mg/l; (c) NAA:TDZ (0.2:0.5) mg/l; (d) NAA:TDZ (0.2:1) mg/l; (e) NAA:TDZ (0.2:3) mg/l; (f) NAA:BAP (0.2:0.5) mg/l; (g) NAA:BAP (0.2:1) mg/l; (h) NAA:BAP(0.2:3) mg/l; (i) NAA:KIN (0.2:0.5) mg/l; (j) NAA:KIN (0.2:1) mg/l; and (k) NAA:KIN (0.2:3) mg/l.