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Effect of Sucrose Excess and Deprivation on the Physiological Responses and Phytochemical Compound Profiles of Kaffir Lime Calli

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Abstract

Background. Kaffir lime (*Citrus hystrix* DC.) calli contain compounds with potential uses in biological activities. The synthesis of these compounds can be enhanced by increasing or decreasing the amount of nutrients. Sucrose provides carbon and energy, which are crucial for the proliferation and development of callus tissue. Sucrose excess or deprivation can generate osmotic potential, which affects the synthesis of bioactive compounds. This study aims to analyze the physiological responses and phytochemical compound profiles of kaffir lime calli due to variations in sucrose concentrations.

Methods. Calli were induced from seed explants using sucrose concentrations of 30 (S1) and 40 (S3) g/L. At the subculture stage, the callus was transferred to a medium with a regular sucrose concentration of 30 g/L (S1), deprivation sucrose concentration of 20 g/L (S2), and excess sucrose concentration of 40 g/L (S3). Observed parameters included biomass, morphology (color and texture), and bioactive compound profiles.

Results. Growth curves indicated that although the groups had the same growth phase patterns, the biomass of G1 calli under the S3–S3 treatment was significantly higher than in other treatments. In addition, the S3–S3 treatment produced the greatest number of compounds, followed by the S1–S2 treatments. This includes the total number of compounds detected by GC-MS and the number of compounds possessing biomedical activities including anticancer, antitumor, antioxidant, anti-inflammatory, antimicrobial, antibacterial, antiviral, and antifungal properties.

Conclusion. Excess and deprivation of sucrose concentration both have effects on kaffir lime calli. Treatment with excessive sucrose (S3 to S3) results in a rise in G1 callus biomass. Meanwhile, treatment with S3 to S3 and S1 to S2 (regular to deprivation) enhances the synthesis of compounds with biomedical activities. This occurs because sucrose stimulates the synthesis of bioactive compounds by serving osmotic stressor and energy source.

Keywords: kaffir lime, callus, sucrose, bioactive compounds, physiological

1. Introduction

Our previous study succeeded in inducing friable and genetically stable calli from kaffir lime (*Citrus hystrix* DC.) seeds (Tunjung *et al*., 2021). These calli contained various bioactive compounds, such as α-pinene and 1,8-cineole, which serve as therapeutic agent (Tunjung *et al*., 2020). The potential in biological activities of kaffir lime calli needs to be developed by increasing their bioactive compound production and maintaining their good growth. One strategy for producing bioactive compounds is the use of plant tissue culture techniques. Through plant tissue culture, the production of bioactive compounds can be enhanced using various strategies such as adding nutrients and growth hormones to the culture medium, adjusting the medium, adding biotic and abiotic elicitors, and introducing precursors. The culture media can be supplemented with macronutrients and micronutrients to provide essential nutrition for the callus, thereby promoting callus growth. Adding specific amounts of nutrients, whether in deficiency or excess, can induce stress, which in turn enhances the synthesis of bioactive compounds (Selwal *et al*., 2023). Incorporating elicitors into the culture medium induces stress conditions that promote the synthesis of bioactive compounds (Laezza *et al*., 2024). The addition of precursors can activate enzymes responsible for the production of bioactive compounds, thus increasing their synthesis rate (Aksenova et al., 2023). For instance, the addition of of glutathione precursors to the culture medium has been shown to enhance cell suspension biomass and organosulfur compound accumulation in *Allium sativum* L. (Setiowati *et al*., 2022). Hence, the addition of precursor, elicitor or nutrient into culture media warrant further investigation.

Sucrose is the predominant carbon source utilized in plant tissue culture. In vitro conditions, added sucrose may play multiple roles as a carbon, energy sources and an osmotic agent that may impose osmotic stress (Dantas *et al*., 2021). The presence of sucrose in culture media

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facilitates cell division, which is essential for the physiology and biomass of calli (Khan *et al*., 2018). Moreover, sucrose participates in terpenoid biosynthesis through the glycolysis cycle, producing precursors in the form of acetyl-CoA, which is shunted to the mevalonate process in cytosolic organelles (Stein and Granot, 2019). In the cytosolic pathway, the mevalonate (MVA) cycle can synthesize monoterpenes in plastid organelles (Yu *et al*., 2015). According to Tholl (2015), 3-hydroxy-3-methylglutaryl-CoA reductase, the primary enzyme in terpenoid biosynthesis, is regulated by the activity of protein kinase SnRK1, which is in turn modulated by glucose as a signal.

At optimal concentrations, sucrose can maintain cell turgor pressure, which is critical in the formation of callus cell structures. Javed, and Ikram, (2008) stated that in the induction of wheat (*Triricum aestivum* L.) calli, a sucrose concentration of as high as 40 g/L in media can impose osmotic pressure and water potential to produce a high biomass weight of 80 mg, while sucrose is 30 g/L produced a biomass weight of 50 mg. At high concentrations, sucrose can act as an abiotic stressor capable of increasing the production of secondary metabolite compounds. A previous study demonstrated that the stem calli of *Wedelia biflora* (L.) D.C exposed to 40 g/L sucrose produced 88 mg of stigmasterol compounds, whereas that exposed to 30 g/L sucrose produced 58.3 mg of stigmasterol compounds (Idris *et al*., 2018). These data emphasize the importance of optimizing sucrose concentration.

In the current study, we investigated the effect of sucrose excess and deprivation on callus growth. At generation 0 (G0), we used two sucrose concentrations: regular and excessive. At the subculture stage (G1), the calli were subjected to deprivation, regular, and excessive sucrose concentrations. This study aimed to analyze the impact of excess and deprivation of sucrose on the physiological responses and phytochemical profiles of kaffir lime calli.

2. Materials and methods

2.1. Sampling

Kaffir lime fruit was collected from Pekutan, Bayan, Purworejo, Central Java, Indonesia. The temperatures at the sample location were 31°C in the air, 30°C in the soil, 676 Cd for light intensity, and 33% for air humidity. The site was located 232 meters above sea level. The citrus fruits used were three months old. Fruit was selected if it had a diameter of 5–6 cm and fresh green color and was not moldy and excessively wrinkled. The seed explant used was 1–2 cm in diameter.

2.2. Induction of kaffir lime calli

Seed sterilization, callus induction, and subculture were performed by using a method from our previous study (Tunjung *et al*., 2021). The media used in this study was Murashige and Skoog medium containing macronutrients ($CaCl_2 \cdot 2H_2O$, KNO_3, KH_2PO_4, NH_4NO_3, and $MgSO_4 \cdot 7H_2O$), iron ($FeSO_4 \cdot 7H_2O$ and Na_2–EDTA), micronutrients ($MnSO_4 \cdot H_2O$ of 22.3 mg/L, $ZnSO_4 \cdot 4H_2O$ of 8.6 mg/L, H_3BO_3 of 6.2 mg/L, $CuSO_4 \cdot 5H_2O$ of 0.025 mg/L, $CoCl_2 \cdot 6H_2O$ of 0.025 mg/L, KI of 0.83 mg/L, $NaMoO_4 \cdot 2H_2O$ of 0.25 mg/L), vitamins, and myo-inositol. The concentration of sucrose varied as follows: 30 g/L (S1) representing the regular concentration, 20 g/L (S2) as the deprivation concentration, and 40 g/L (S3) as the excess concentration. Media were added with a plant growth regulator in the form of 2,4-D:BAP at a ratio of 1:0.5 ppm. Kaffir lime seeds were sterilized with 5.25% NaOCl. The sterile kaffir lime seeds were cut and placed in an incubation room in the dark.

2.3. Subculture of kaffir lime

G0 calli were induced on a medium with sucrose concentrations of 30 g/L and 40 g/L. The calli were weighed every five days to determine their growth phase. Additionally, the texture and color of the calli were analyzed. Subcultures (G1) were initiated when the calli reached the exponential phase, approximately 25–30 days after induction. The calli were then cut into two pieces, each measuring 0.5 cm, and exposed to varying sucrose concentrations in the new medium: 30 g/L (S1), 20 g/L (S2), and 40 g/L (S3). Morphology and growth phase measurements were performed every five days until day 50.

Table 1. Sucrose concentration

Treatment Group	Sucrose concentration (g/L)	
	Generation 0	Generation 1
S1–S1	30	30
S1–S2	30	20
S1–S3	30	40
S3–S1	40	30
S3–S2	40	20
S3–S3	40	40

Notes: S1-S1: regular sucrose concentration to regular sucrose concentration (30 g/L to 30 g/L)

S1-S2: regular sucrose concentration to deprivation sucrose concentration (30 g/L to 20 g/L)

S1-S3: regular sucrose concentration to excess sucrose concentration (30 g/L to 40 g/L)

S3-S1: excess sucrose concentration to regular sucrose concentration (40 g/L to 30 g/L)

S3-S2: excess sucrose concentration to deprivation sucrose concentration (40 g/L to 20 g/L)

S3-S3: excess sucrose concentration to excess sucrose concentration (40 g/L to 40 g/L)

2.4. Physiological and growth parameters of kaffir lime calli

G1 Calli were weighed every 5 days over a 50-day period to construct growth curves. A total of 9 calli were measured at each interval. The wet and dry weights of the calli were recorded, with the dry weight obtained after drying the calli in an oven at 33°C for 7 days. The growth curves were used to identify the lag, exponential, and stationary phases of growth. The lag phase is characterized by minimal changes in callus biomass at the onset of induction. The exponential phase is marked by a substantial increase in callus biomass. The stationary phase is characterized by relatively stable callus biomass. The morphological indices for measurement and observation included callus texture and color. The texture of the observed calli was determined as friable or compact. Calli texture is classified as follows: callus initiation with compact texture (+), callus beginning to spread with

compact texture (++), callus starting to dominate the explant with friable texture (+++), and callus fully dominating with friable texture (++++). The color of calli was determined on the basis of the Royal Horticultural Society color chart.

2.5. Extraction

Calli were harvested at stationary phase and dried until they reached a constant weight. A total of 1 gram dry callus powder was macerated in ethyl acetate for 24 h and remacerated three times. Extracts were analyzed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890A system equipped with an Agilent 5977B GC/MS detector (Agilent Technologies, Palo Alto, CA, USA). The MS column used was DB-5MS (5% [phenyl]-methylpolysiloxane). The NIST 16 library database was employed for compound identification.

2.6. Statistical analysis

One-way ANOVA was performed using SPSS software to examine both wet and dry weights of G1 callus at t 30 days, which is the maximum biomass. In order to find significant variations in the treatment medium, Duncan's Multiple Range Test (DMRT) was then run at a 5% significance level.

3. Result

3.1. Callus growth

The G1 growth curves of the kaffir lime calli over 50 days are shown in Figure 1.

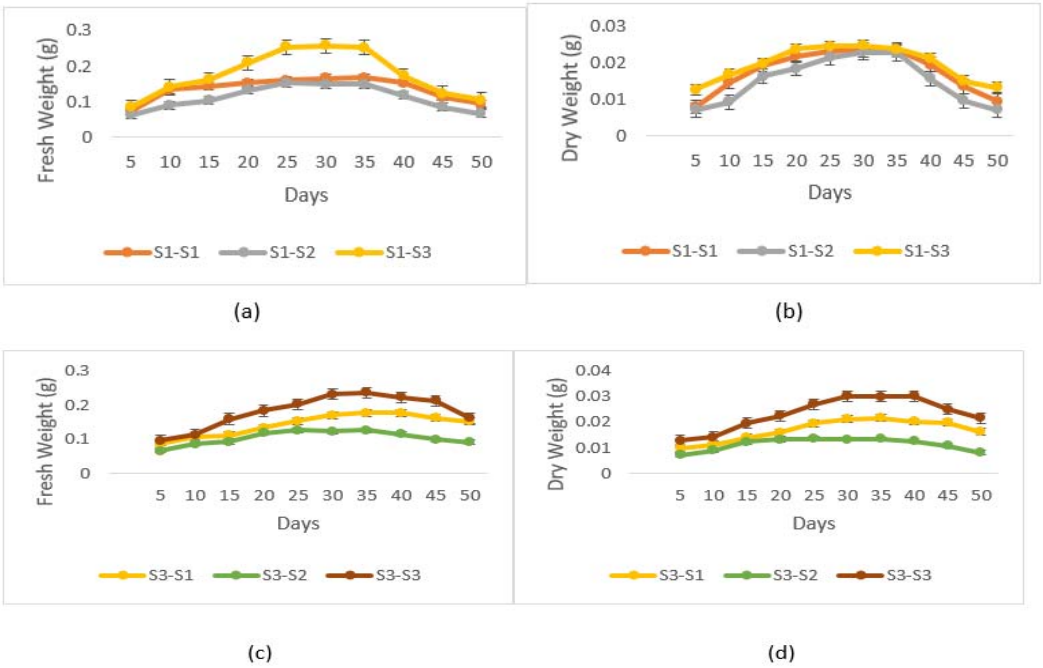


Figure 1. Growth curves of kaffir lime calli. Under deprivation sucrose (a Fresh Weight b. Dry Weight) and excessive sucrose concentration treatment (c. Fresh Weight d. Dry Weight).

The callus growth curve indicated that the biomass of calli under the S1 treatment did not differ significantly, as S1 represents the standard sucrose dose used in Murashige and Skoog medium. In contrast, calli under the S3 treatment showed different biomass weights. The S3–S3 (excess–excess) treatment showed the significant highest biomass, while the lowest was found under the S3–S2 (excess–deprivation) treatment (Figure 2). The striking difference in sucrose concentration between G0 and G1 may have caused the reduction in callus biomass.

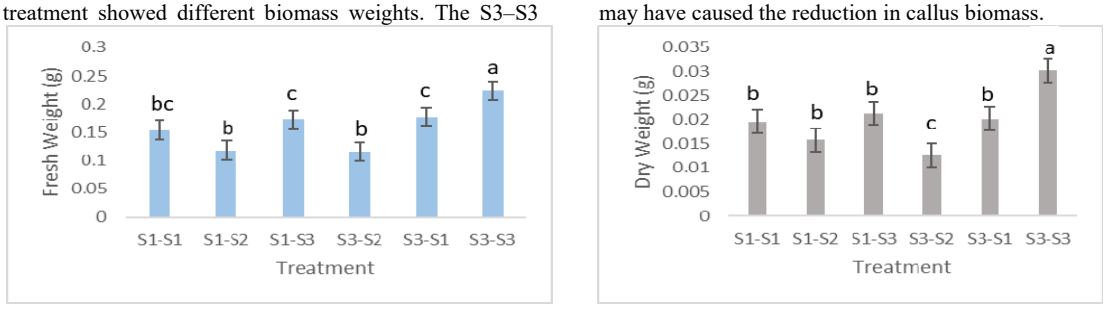


Figure 2. Statistical analysis of G1 Kaffir lime calli. Fresh weight (a) and dry weight (b). Significant different are shown by different letters, according to an ANOVA with a significance level of $\alpha < 0.05$.

Table 2 presented the growth phases of G1 calli based on their growth curves.

Table 2. Growth phase of G1 kaffir lime calli under various sucrose treatments

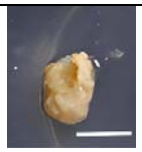
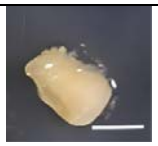
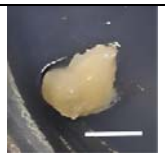
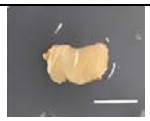
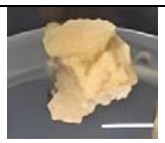
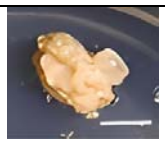
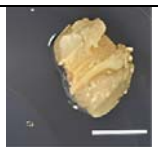
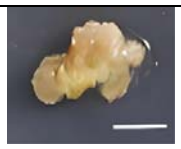
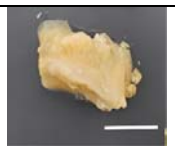
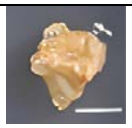
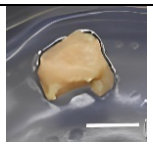
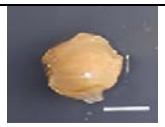
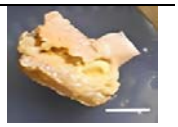
Treatment	Growth phase (day)		
	Lag phase	Exponential phase	Stationary phase
S1–S1	5–10	10–30	30–40
S1–S2	5–10	10–30	30–40
S1–S3	5–10	10–30	30–40
S3–S2	5–10	10–30	30–40
S3–S1	5–10	10–30	30–40
S3–S3	5–10	10–30	30–40

Furthermore, no differences were found in the growth phase of each group. Days 5-10 were the lag phase, days 10-30 were the exponential phase, and days 30-40 were the stationary phase. However, callus biomass significantly differed among the treatment groups. Given its dual roles as an osmotic regulator and as a nutrient, which is vital for the embryoid and callus formation, sucrose has the potential to impact callus biomass. Calli must be grown in a controlled media with 30 g/L sucrose (Thaneshwari, 2018). Therefore, excess or deprivation of sucrose will affect callus biomass but not the growth phase of the callus in each sucrose treatment group.

3.2. Physiological responses of kaffir lime calli

The morphological characteristics of kaffir lime calli, including color and texture, are illustrated in Table 3.

Table 3. Color and texture of G1 kaffir lime calli exposed to various sucrose concentrations

Days	S1–S1	S1–S2	S1–S3	S3–S2	S3–S1	S3–S3
Day 10 (Lag phase)						
	Pale yellow: 161C	*Pale greenish yellow*: 160C	*Pale yellow*: 160D	*Pale yellow*: 161C	*Pale yellow*: 161C	*Pale yellow*: 160D
	+++	+++	++++	++++	++++	++++
Day 30 (Exponential phase)						
	Light yellow: 162 C	*Moderately yellow*: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A
	++++	++++	++++	++++	++++	++++
Day 40 (Stationary phase)						
	Moderately yellow: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A	*Moderately yellow*: 161A
	++++	++++	++++	++++	++++	++++

Notes:

S1: 30 g/L, S2: 20 g/L, S3: 40 g/L

(−) : Callus has not yet appeared

(+): Callus initiation

++: The callus has begun to spread throughout the explant and has a compact texture

+++: The callus has begun to dominate the explant and has a friable texture

++++ : The callus has dominated the whole section and has a friable texture.

The scale bar represents a size of 1 cm

In this study, varying sucrose concentrations did not cause differences in callus color and texture. This result proves that sucrose excess and deprivation lacked an effect on callus color or texture.

3.3. Compound profiles of kaffir lime calli

Kaffir lime calli exposed to various sucrose concentrations can be used to biosynthesize bioactive compounds. The percentage of peak area, and names of bioactive compounds are shown in Table 4. The percentage of peak area (%) reflects the relative proportion of each detected compound within a sample, highlighting the abundance of each compound in comparison to the total mixture.

Table 4. Bioactive Compounds in Calli Possess Biomedical Activity

No.	Compound	The percentage of peak area in the sucrose treatment (%)						Group	Biomedical activity
		S1–S1	S1–S2	S1–S3	S3–S2	S3–S1	S3–S3		
1	2-Hexanone, 3-methyl-4-methylene	0.42	ND	ND	ND	0.86	ND	Acetic acid hexyl ester	AO, AM (Karthik et al., 2023)
2	*sec*-Butyl nitrite	0.36	ND	ND	1.43	ND	0.07	Heptadecane	AO (Ghasemi et al., 2023)
3	1-Hexyn-3-ol	0.39	ND	ND	ND	1.46	0.29	Alcohol-ketone	AB (Charles et al., 2018)
4	2,4-Nonadienal	0.16	0.21	ND	0.28	ND	0.22	Aldehyde	AF, AO (Bourhia et al., 2021)
5	Heptane, 2,4-dimethyl-	0.1	ND	ND	0.13	ND	ND	Acyclic short-chain alkane	AF, AB (Al-Rahbi et al., 2023)
6	1-Decene, 2,4-dimethyl-	0.56	ND	0.41	ND	ND	ND	Alkene	AC, AO (Al-Mansoub et al., 2021)
7	Naphtalene	5.22	5.5	5.78	4.13	3.77	5.94	Sesquiterpene	AO (Ozen et al., 2018)
8	3-Hexanone, 2,5-dimethyl-	0.16	0.22	ND	0.45	ND	ND	Isobutyl ketone	AM (Karthik et al., 2023)
9	1-Octanol, 2-butyl-	0.2	3.49	ND	3.71	ND	1.47	Aliphatic alcohol	AM, AC (Muzahid et al., 2023)
10	Dodecane, 4,6-dimethyl-	1.17	7.6	ND	1.24	1.44	ND	Alkane	AO and AM (Wiraswati et al., 2023)
11	2-Decenal	0.47	ND	ND	0.89	ND	ND	Unsaturated aldehyde	AM (Wiraswati et al., 2023)
12	Oxalic acid and allyl pentadecyl ester	0.53	0.74	ND	ND	0.28	0.35	Ester	AF, AM (Doughari and Abraham, 2021)
13	1-Undecene, 7-methyl-	3.44	4.11	1.71	4.45	ND	ND	Volatile oil	AO, AI, AB, and AF (Muzahid et al., 2023)
14	Octan-2-one, 3,6-dimethyl-	0.78	0.32	0.33	ND	ND	ND	Branched ketone	AM (Wang et al., 2022)
15	Heptadecane, 2,6,10,14-tetramethyl-	0.31	0.41	ND	ND	ND	ND	Hydrocarbon-alkane	AC (Zare et al., 2023)
16	Decane, 2,3,5,8-tetramethyl-	0.49	1.56	0.73	1.88	ND	2.55	Aliphatic hydrocarbon	AO, AC (Avidlyandi et al., 2021)
17	Diethyl phthalate	0.89	1.01	0.75	2.78	1.37	2.37	Phthalate ester	AO (Amara et al., 2020)
18	2-Piperidinone, *N*-[4-bromo-*n*-butyl]-	0.09	1.07	0.28	0.97	0.29	0.96	Alkaloid	AM (Al-Salman 2019)
19	2,5-Pentadecadien-1-ol	0.24	ND	ND	0.3	ND	0.47	Unsaturated alcoholic compound	AB, AI, AO (Chrząszcz et al., 2023)
20	1,4-Bis(trimethylsilyl)benzene	0.27	0.45	ND	1.99	0.25	0.9	Trimethylsilyl	AO (Wiraswati et al., 2023)
21	1,2-Bis(trimethylsilyl)benzene	0.28	0.64	ND	0.28	0.5	0.91	Trimethylsilyl	AM (El-Zawawy and Mona 2021)
22	1-Undecene, 4-methyl	0.5	0.64	ND	ND	ND	ND	Hydrocarbon	AM (Tleubayeva et al., 2021)
23	2-Heptenal,	ND	0.64	0.28	ND	ND	ND	Unsaturated fatty acids	AT, AV, AI (Gu, et al., 2019)
24	2-Decene	ND	1.09	0.55	ND	ND	ND	Unsaturated aldehyde	AM (Lee et al., 2022)
25	2,4-Decadienal	ND	0.8	0.22	ND	ND	ND	Unsaturated aldehyde-aliphatic aldehyde	AB (Zhang et al., 2020)
26	d-Glycero-d-ido-heptose	ND	0.42	ND	ND	ND	0.9	Sugar	AB (Guo et al., 2021)
27	Cyclotrisiloxane, hexamethyl-	ND	1.31	1.31	11.97	1.47	17.02	Fatty acid-phenolic compound	AM and AO (Gheda and Ismail 2020)
28	Palmitic acid	ND	0.61	20.43	ND	0.79	ND	Saturated fatty acids	AC, AI (Zhu et al., 2021)
29	1-Octyn-3-ol	ND	ND	ND	0.32	ND	0.08	Octynol-alkyne	AM (Xiong et al., 2017)

No.	Compound	The percentage of peak area in the sucrose treatment (%)						Group	Biomedical activity
		S1–S1	S1–S2	S1–S3	S3–S2	S3–S1	S3–S3		
30	Cyclohexane, 1,1,2-trimethyl	ND	ND	ND	0.34	ND	0.53	Fatty acyls-hydrocarbon	AO (Onanuga et al., 2022)
31	Oxirane, 2-butyl-3-methyl-	ND	ND	ND	0.22	ND	0.24	Epoxide heptane	AM (Njoroge et al., 2019)
32	4-Tridecene	ND	0.17	ND	ND	0.17	ND	Fatty acyls-hydrocarbon	AM (Lammers et al., 2021)
33	E-10-Dodecen-1-ol propionate	ND	ND	ND	0.26	ND	0.63	Lipid	AO, AC, AM (Paudel et al., 2020)
34	4-Ethyl-1-hexyn-3-ol	ND	ND	ND	ND	1.46	0.29	Alcohol-ketone	AB (Charles et al., 2018)
35	Tridecane	ND	ND	ND	ND	1.33	3.03	Alkane	AC (Singh and Luqman 2014)
36	Tetradecane, 1-fluoro	ND	ND	ND	ND	0.22	0.92	Tetradecyl fluoride	AB, AM (Nasr et al., 2022)
37	Decane, 2,4,6-trimethyl	ND	ND	ND	ND	0.45	1.1	Alkane hydrocarbon	AO, AM (Wiraswati et al., 2023)
38	Citronellol epoxide	ND	0.52	ND	ND	ND	ND	Derivative terpene epoxide	AC (Ho et al., 2020)
39	9-12, Octadecanoic acid	ND	ND	0.41	ND	ND	ND	Linoleic acid ester	AC, AI (Gheda and Ismail 2020)
40	1-Iodo-2-methylnonane	ND	ND	ND	ND	ND	0.1	Iodine compound	AO (Khan et al., 2021)
41	Dodecanoic acid, 3-hydroxy	ND	ND	ND	ND	ND	1.12	Ethyl ester fatty acid	AC (Ukwubile et al., 2019)

Bioactive compounds related to anticancer effects

Bioactive compounds related to anticancer and antimicroorganism effects

Bioactive compounds related to antimicroorganism effects

Notes: The percentage of peak area (%) reflects the relative proportion of each detected compound within a sample, highlighting the abundance of each compound in comparison to the total mixture. ND = not detected, AC = anticancer, AI = anti-inflammatory, AO = antioxidant, AT = antitumor, AM = antimicrobial, AB = antibacterial, AF = antifungal, AV = antivirus.

The secondary metabolite profiles of calli differed in terms of the types of compounds produced under each treatment. Common compounds produced under almost all treatments included alkanes, such as butane, 1-chloro-3-methyl, and dodecane,4,6-dimethyl-; alkaloids, such as 2-periperidinone and *N*-[4-bromo-*n*-butyl]; fatty acids, namely, 9-octadecenoic acid (*Z*)-, phenylmethyl ester; and terpenoid compounds, specifically, the sesquiterpene naphthalene. The percentage of the peak area of naphthalene compounds, which have anticancer activity, decreased in calli that experienced sucrose excess during G0 and were subcultured on deprivation or regular medium during G1.

Calli grown on regular medium during G0 produced several types of compounds, including aldehydes, such as 2-decenal and 2,4-decadienal; ketones, namely, octan-2-one and 3,6-dimethyl-; fatty alcohols, such as 11-methyldodecanol; fatty acids, including palmitic acid; and monoterpenoids, namely, citronellol epoxide. These compounds were not found in calli grown under sucrose excess during G0. Sucrose excess during G0 resulted in the production of high amounts of compounds. These compounds included alkanes, such as decane, 2,4,6-trimethyl, tridecane, and hexadecane; fatty alcohols, including 1,2:4,5:9,10-triepoxydecane; fatty acids, such as dodecanoic acid and 3-hydroxy; and flavonoids, namely, 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione.

Table 5. Summary of Bioactive Compounds Detected in Kaffir Lime Calli

Sucrose concentration	Number of compounds					
	S1–S1	S1–S2	S1–S3	S3–S2	S3–S1	S3–S3
Total peak detected in GCMS	45	44	29	44	39	52
Total compounds Possess Biomedical Activity	22	23	13	20	16	24
Bioactive compounds related to anticancer effects (AC, AT, AO &AI)	13	13	9	13	9	15
Bioactive compounds related to antimicroorganism effects (AM,AB,AF &AV)	15	16	6	14	11	15

Notes: AC = anticancer, AI = anti-inflammatory, AO = antioxidant, AT = antitumor, AM = antimicrobial, AB = antibacterial, AF = antifungal, AV = antivirus

According to GCMS data, a total of 29 compounds with low peaks were detected under the S1–S3 treatment, whereas 52 compounds with high peaks were found under the S3–S3 treatment. The number of peaks indicates the variety of compounds detected in the callus. Among these detected compounds, we investigated which bioactive substances exhibit biomedical activity and hold potential for development as traditional medicines. The S3–S3 sucrose treatment produces the highest number of compounds with biomedical activity, followed by the S1–S2 treatment. These results may be due to the effect of sucrose concentrations on osmotic potential; both high and low concentrations can reduce osmotic potential, leading to stress in the calli and the subsequent synthesis of secondary metabolites (De Paiva and Otoni, 2003). Additionally, the S3 treatment was applied during G0 (callus induction), causing the calli to experience stress from the beginning of the experiment.

4. Discussion

In this study, sucrose was added at various concentrations (regular, deprivation, and excess) to kaffir lime calli. Carbohydrates are essential for plants to survive. They serve as a substrate for respiration, function in the synthesis of numerous chemicals, and constitute macromolecule building blocks (Wahyuni *et al*., 2020). Furthermore, carbohydrates may regulate several cellular developmental processes (Dantas *et al*., 2021). Sumaryono et al. (2012) reported that sucrose is the most often utilized carbohydrate source in plant tissue culture because disaccharides are widely used as transporter molecules likely due to their high water solubility. The majority of in vitro studies have shown that sucrose supports growth and can enhance cell biomass (Al-Zubaidy *et al*., 2020). The development of somatic embryos in culture medium is influenced by the amount of sugar concentration (Lema-Ruminska *et al*., 2013).

In this current study, the highest dry and fresh weights of G1 calli were observed in the S1-S3 and S3-S3 (excessive) treatments. The S3-S3 treatment produced significantly higher biomass, whereas the biomass in the S1-S3 treatment was not significantly different from that in the S1-S1 and S1-S2 treatments. The lowest biomass was observed in the S3-S2 (deprivation) treatment. Therefore, among the treatments, exposure to excessive sucrose during G0 or G1 is the best for increasing callus biomass. This result is related to the role of sucrose sucrose as an energy-providing carbon substrate for plant growth in vitro (Homsuwan *et al*., 2021). Sucrose is an essential component of medium for in vitro plant development because it supplies organic nutrients and controls osmotic pressure (Liang *et al*., 2018). The breakdown of sucrose into fructose and glucose increases the osmolality of media. Sucrose concentration can impose osmotic potential on the enlargement of callus cells (Sumaryono *et al*., 2012). It can impose osmotic stress on calli and result in proline accumulation, which is useful for overcoming the effects of osmotic stress due to the total loss of water and carbohydrates (Gerdakaneh *et al*., 2010; Hayat *et al*., 2012). Therefore, the presence of osmotic stress can cause the fresh weight of calli to increase. This situation indicates that calli contain water and carbohydrates (Sari *et al*., 2018).

In cellular metabolism, glucose and fructose, as constituents of sucrose, enter the glycolysis pathway and Krebs cycle to form ATP, which is needed for callus growth. Gerdakaneh et al. (2010) reported that differences in dissolved sucrose concentrations cause variations in turgor and osmotic pressure. In tissue culture, turgor pressure affects the elongation of callus cells. In addition, each cell exhibits different growth responses to changes in turgor pressure due to the addition of sucrose. Moreover, because sucrose promotes cell respiration, which increases callus biomass, its addition accelerates callus growth. Meanwhile, low callus biomass can occur under deprivation treatment because of the response of callus cells to the lack of sucrose as the main energy source for callus growth. Therefore, callus growth is not optimal. The rate of respiration and absorption of nitrogen reduce under low sucrose concentrations. Protein synthesis is therefore may be inhibited as a result of the decrease in available energy (Thaneshwari, 2018).

Morphological characteristics in terms of color or texture showed almost no differences between calli exposed to sucrose deprivation or excess. All G1 calli had a yellowish color and tended to be pale with a friable texture. Callus color is influenced by the color of the explant, namely, the seed. The absence of differences in callus color and texture among treatments indicates that the addition of sucrose does not affect callus morphology.

High (excess) and low (deprivation) concentrations of sucrose affect the amount and type of bioactive compounds produced. Phenylalanine ammonia-lyase (PAL), which can provide precursors for the total biosynthesis of phenolics and flavonoids, may become more active when sucrose is added (Jaafar *et al*., 2012). In the phenylpropanoid pathway, PAL is the main enzyme. It catalyzes the transformation of *trans*-cinnamic acid from L-phenylalanine, which is generated via the shikimate pathway. The phenylpropanoid pathway can convert *trans*-cinnamic acid into intermediate molecules (sinapic and coumaric acids) that can be further transformed into coumarin and chlorogenic acid, which can then be further transformed into secondary metabolites (Sharma *et al*., 2019). These compounds have biological activities including antioxidant, anti-inflammatory, and anticancer activities. Another study revealed that sucrose stimulates anthocyanin biosynthesis in *Arabidopsis thaliana* by upregulating both structural genes and positive transcription factors associated with the flavonoid biosynthesis pathway. Additionally, sucrose may facilitate this process by simultaneously downregulating the negative transcription factor MYBL2 (Kyoko *et al*., 2008). These findings support the study results, which show that the S3–S3 treatment produced the highest total peak and the greatest number of compounds possesses biomedical activity among all treatments. Most of these compounds exhibit anticancer, anti-inflammatory, antioxidant, antitumor, antimicrobial, antibacterial, antifungal, and antiviral activities. This result suggests that high concentrations of sucrose (excessive) are suitable for the production of compounds with biomedical activity.

The S1–S2 deprivation treatment can also potentially stimulate the synthesis of various biomedical compounds, including antibacterial, antimicrobial, and antifungal agents because it causes calli to become stressed due to nutritional deficiencies. It can trigger calli to produce

bioactive compounds because carbohydrates accumulate as signals and provide energy for the synthesis of various chemicals (Lee and Huang, 2014).

Several bioactive compounds were observed under all treatments. They included napththalene, a sesquiterpene; diethyl phthalate, a phthalate ester, and 2-piperidinone, an alkaloid. In addition, two types of terpenoid compounds were detected in calli: naphthalene and citronellol epoxide. Naphthalene compounds had the highest peak area of 5.94% under the S3–S3 treatment, whereas citronellol epoxide was found under the S1–S2 treatment with a peak area of 0.52%. Citronellol epoxide is a citronellol compound resulting from the epoxidation process thus changing the structure of the compound. Peel and leaf of *Citrus* species including *Citrus maxima*, *Citrus limonia*, *Citrus limon*, and *Citrus reticulata* are typical sources of the chemical citronellol (Das *et al*., 2024), and we could find this compound in kaffir lime's calli

Meanwhile, the first to third highest percentage peak areas in all the detected compound profiles were shown by palmitic acid (S1–S3), cyclotrisiloxane (S3–S3), and dodecane,4-6dimethyl (S1–S2) with peak percentages of 20.43%, 17.02%, and 7.6%, respectively. The highest peak of most of the mentioned compounds occurred under the S3 treatment during G0 and G1 because exposure to high sucrose concentrations before subculture provided calli with sufficient nutrients during G0. Some compounds showed the highest peak or appeared only under the S2 (deprivation) treatment. Sucrose deprivation can induce nutritional stress in calli such that they synthesize bioactive compounds. Liang et al. (2018) provide evidence for this assumption by describing how 20 g/L sucrose may be utilized to ensure that Chinese Kale "Cutiaoyusun" hypocotyls promote an excellent proliferation rate (213.5%) of calli.

Treatment with excess sucrose (S3–S3) also increased the peak areas of several types of compounds with biomedical activity. These compounds included naphthalene; decane, 2,3,5,8-tetramethyl-; cyclotrisiloxane, hexamethyl-; cyclohexane, 1,1,2-trimethyl; E-10-dodecen-1-ol propionate; tridecan; tetradecane, 1-fluoro; and decane, 2,4,6-trimethyl. 1-Iodo-2-methylnonane and dodecanoic acid, 3-hydroxy with anticancer and antioxidant activities were found only under the S3–S3 treatment. These compounds have various roles in the medical field. For example, naphthalene compounds have anticancer activity. A novel family of chalcone derivatives with IC_{50} values of 1.13 ± 0.08, 0.82 ± 0.11 μM, and 0.65 ± 0.06 respectively, showed high cytotoxic action against HCT116, MCF-7, and HepG2 cells in earlier investigations. These compounds included indole and naphthalene groups. Their anticancer method is predicated on blocking the advancement of the cell cycle and tubulin polymerization (Wang *et al*., 2019). In addition, another study revealed that the compound cyclotrisiloxane, hexamethyl- was present in *Acacia karoo* leaf extract obtained with ethyl acetate solvent. The extract had a large inhibition zone of 33 ± 1.53 mm against *Staphylococcus aureus* (Priyanka *et al*., 2014).

Accordingly, the S1–S2 and S3–S3 treatments were found to be effective in enhancing biomass, physiological responses, and phytochemical compound synthesis in calli. This is likely because sucrose fulfills the nutritional requirements of calli. Existing nutrients can impose osmotic pressure, which can increase water adsorption and carbohydrates. Adequate nutrition can also support cell division in calli such that calli under different treatments were heavier than those under the S1 and S3 treatments.

Therefore, different treatments must be selected to increase the growth of calli and amount of bioactive compounds. For callus growth, sucrose must be added for energy. For bioactive compound synthesis, sucrose excess (S3–S3) or deprivation (S1–S2) must be imposed to meet nutritional needs and induce nutritional stress.

5. Conclusion

Treatment with various sucrose concentrations affects callus growth and morphology. Among the treatments, S3–S3 during the G1 phase resulted in the highest fresh and dry callus biomass. Treatments with excessive sucrose concentrations (S3–S3) and deprivation sucrose concentrations (S1–S2) are effective in enhancing the production of compounds with biomedical activity. This is because sucrose supplies energy and induces osmotic stress on the callus, which stimulates the synthesis of these compounds.

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