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Cellular Growth Dynamics Affects Allelopathic Activity in Coffee Cell Culture

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Abstract

Cellular growth dynamics and allelopathic activity in coffee cell cultures were examined as follows: First, we compared allelopathic activity of seven woody plant calli, *Coffea canephora*, *Derris indica*, *Ficus carica* L., *Juniperus conferta*, *Prunus persica*, *Punica granatum*, and *Sonneratia ovata*, using a modified "sandwich method bioassay" and found that coffee callus showed the strongest growth inhibition to lettuce seedling nearly 90% of hypocotyl and 96% of root. This coffee callus actively proliferated, with a 21-fold increase during five weeks of subculture, with a growth curve comprising two typical phases: a lag phase of 0 - 2 weeks of culture and an exponential phase of 3 - 5 weeks of culture. Allelopathic activity varied depending on the growth phase of the coffee callus. The strongest allelopathic activity was detected in 1 - 2-week-old callus showing nearly 100% inhibitory effect on lettuce seedling growth. As the allelopathic activity of coffee calli is extremely high, beyond the natural level in coffee leaves and green beans, we focused on analyzing the allelopathic activity of its aqueous extracts using high-performance liquid chromatography. Several prominent peaks, including two reference alkaloids, theobromine and caffeine, which are known allelochemicals in coffee plants, and three distinct unknown peaks were identified at 270 nm in coffee calli during the lag phase (1 - 2 weeks of culture). The higher value of the total phenolic content in the lag phase also suggested a key biosynthetic pathway in relation to the allelopathic activity of coffee callus will be activated in the lag phase.

Keywords

Allelopathic Activity, *Coffea canephora*, Callus, Growth Phase, Sandwich Method Bioassay

1. Introduction

Allelopathy refers to the metabolic ability of a plant to release bioactive chemicals into its surrounding environment, which affects the growth, germination, and development of other plants. The inhibition of germination and early growth of seedlings is commonly observed as the main allelopathic effect [1]. Since the sandwich (SW) method, a small-scale bioassay, was established [2] [3], it has been widely used to determine the allelopathic potential of many plants, including bryophytes [4], transgenic aspen and eucalyptus [5], 178 Caucasian plant species [6], and medicinal plants [7].

The optimization of cell culture systems is a promising approach for unveiling sequential metabolic processes in plants of interest. Several studies on the allelopathic activity in plant cell cultures have been published, including those on small everlasting [8], asparagus [9], leguminous mangrove plants [10], and bamboo plants [11].

We established a unique coffee cell culture system, Robusta coffee *(Coffea canephora*) non-embryogenic calli (nEC), which showed a strong inhibitory effect on lettuce cell division [12] and seedling growth [13]. In this study, we evaluated the allelopathic activity of coffee calli using the SW method. Six prominent calli of conifer trees (Jc, *Juniperus conferta*), fruit trees (Fc, *Ficus carica* L., Pg, *Punica granatum*, Pp, *Prunus persica*), pongame oil tree (Di, *Derris indica*), and mangrove trees (So, *Sonneratia ovata*) were prepared, and their allelopathic activity was compared with that of coffee calli. We further examined the growth curve during maintenance subculture and suggested two typical phases: the lag phase (1 - 2 weeks of culture) and the exponential phase (3 - 5 weeks of culture). The relationship between cellular growth dynamics and allelopathic activity was discussed by adapting the SW bioassay, high-performance liquid chromatography (HPLC) analysis, and the Folin-Ciocalteu method.

2. Materials and Methods

2.1. Coffee Callus Culture

In this study, we used the nEC of the Robusta coffee (Cc, *Coffea canephora*), as shown in **Figure 1** [12]. Regular subculture was performed at 4-week intervals on modified MS medium containing 30 g/L sucrose and 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) to maintain callus quality and viability. The pH of the medium was adjusted to 5.7 before autoclaving. All the cultures were maintained at 25˚C, under the dark condition.

2.2. Sandwich Method Bioassay for Woody Plant Calli

We investigated the allelopathic activity of Robusta coffee callus (Cc) using a modified SW bioassay. Briefly, 10 mg oven-dried (60˚C) coffee callus tissue (4-week-old) and five lettuce seeds (*Lactuca sativa* L., Great Lakes 366) were placed on the test agar medium (0.5% w/v) in a well of six-well plate, as described previously [13]. The test plate was incubated at 20˚C for three days in the

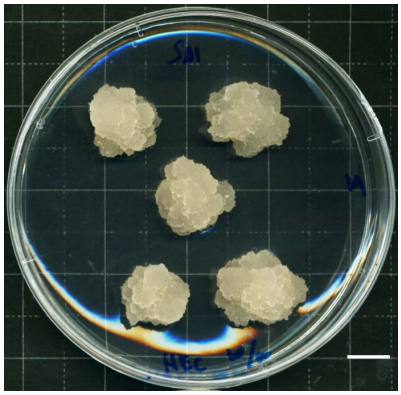

Figure 1. Morphological characteristics of Robusta coffee (*C. canephora*) nEC. Scale indicates 1 cm.

dark. The growth of each lettuce hypocotyl and root was measured using ImageJ software. Likewise, six cultured cell lines from various woody plants, *i.e.*, conifer tree (Jc, *Juniperus conferta*), fruit trees (Fc, *Ficus carica* L., Pg, *Punica granatum*, Pp, *Prunus persica*), pongame oil tree (Di, *Derris indica*), and mangrove tree (So, *Sonneratia ovata*), were also prepared and their allelopathic activity was compared with the value of coffee cultured cells.

2.3. Assessment of Cellular Growth Dynamics and Allelopathic Activity

To determine the impact of cellular growth dynamics on the allelopathic activity of coffee nEC, we investigated the following parameters:

1) Fresh weight increasement: 100 mg fresh weight of callus was transferred to the subculture medium, and subsequent fresh weight measurements were performed using an analytical balance at weekly intervals for 5 weeks.

2) Dry weight (dw)/fresh weight (fw) ratio: After measuring the fresh weight of callus samples as a parameter, they were placed in a drying oven at 60˚C for 18 h. The dw/fw ratio was calculated to provide insights into water content and biomass accumulation, which are indicators of cell growth and physiological changes.

3) Assessment of allelopathic activity: Oven-dried callus samples were collected for 5 weeks, and the SW bioassay was performed as described above.

2.4. Allelopathic Activity of Coffee Leaves and Green Beans

To understand the natural level of allelopathic activity in coffee plants, fresh mature leaves of three coffee species, *C. arabica* (Arabica), *C. canephora* (Robusta), *C. liberica* (Liberica), were collected from the Hiroshima Botanical Garden. The collected leaves were then dried using the original SW method [2]. The dried green beans of these coffee species were ground to a crumble-like texture. All dried samples were weighed at 10 and 50 mg and used for the SW bioassay. By comparing the allelopathic activity between calli and the original plant, we demonstrate the potential of using coffee cell cultures for future allelopathic studies.

2.5. HPLC Analysis

Callus samples were extracted with distilled water at a ratio of 0.2 w/v (100 mg fresh weight and 0.5 mL distilled water) in a microcentrifuge tube, and the supernatant was subjected to reverse-phase HPLC (column: InertSustain C18 5 um 4.6 mmID × 250 mm [GL Sciences, Tokyo, Japan]; column oven: 40˚C; solvent: 20% methanol; flow rate: 0.6 $mL \cdot min^{-1}$; detection: 270 nm). Using a micro syringe (Hamilton, Reno, NV, USA), the injection volume was set to 10 µL. Theobromine (Tb; MW 180.17; Tokyo Chemical Industries, Ltd., Osaka, Japan) and caffeine (Cf; MW 194.19; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used as standards.

2.6. Folin-Ciocalteu Method

This method was originally developed as in [14] and a new approach was introduced as in [15]. The total phenolic content (TPC) of the aqueous extracts was determined by UV-Vis spectroscopy using the Folin-Ciocalteu method. The absorbance was measured at 765 nm using a UV-Vis spectrophotometer. A control sample was prepared using distilled water following the same procedure. A calibration curve was constructed using gallic acid (10 - 50 $mg \cdot mL^{-1}$). All analyses were performed in triplicates.

2.7. Statistical Analysis

The results were presented as the mean + standard deviation (SD) and were subjected to one-way analysis of variance, to determine significant differences between the experimental group, followed by DMRT, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Allelopathic Activity of Seven Woody Plant Calli

We identified the allelopathic activity of the calli of the seven woody plants using a SW bioassay (**Figure 2**). Cc had a significantly stronger inhibitory effect, with an average of approximately 90% of the lettuce hypocotyl growth. A slight inhibitory effect was observed in Jc. A moderate inhibitory effect of 60% was also observed in Pg and Di, whereas Fc and So did not show any inhibitory effects on the lettuce hypocotyls.

All samples showed a clear inhibitory effect on lettuce root growth (>50%). The strongest inhibitory effect was found in Cc, with >95% inhibition, followed by Di, with >90%.

3.2. Cellular Growth Dynamics and Allelopathic Activity in Coffee nEC

Coffee nEC showed a dynamic proliferation capacity during the five weeks of observation (**Figure 3(a)**). Until the second week, coffee nEC showed a 3-fold increase from the initial weight, whereas the increase reached 9-fold in the third

week. At the end of the fifth week of observation, the weight increased by up to 21-fold. The growth curve comprised two typical phases: the lag phase (1 - 2 weeks of culture) and the exponential phase (3 - 5 weeks of culture).

The dw to fw ratio in Figure 3(b) represents moisture content and callus biomass. In the first and second weeks, the dw/fw ratio was the same at 0.045, which was the highest ratio and was significantly different from that in the subsequent culture period. In the third, fourth, and fifth weeks, the ratios were not significantly different, with the lowest ratio of 0.027 in the fifth-week sample.

The SW method (Figure 4) for assessing allelopathic activity showed a strong inhibitory effect on callus samples from the lag phase. The inhibitory effect on the hypocotyl and lettuce roots was nearly 100% in the lag phase samples. During other culture periods, callus samples also still showed a strong inhibitory effect, particularly in the third- and fourth-week samples, with an inhibitory effect of >90%.

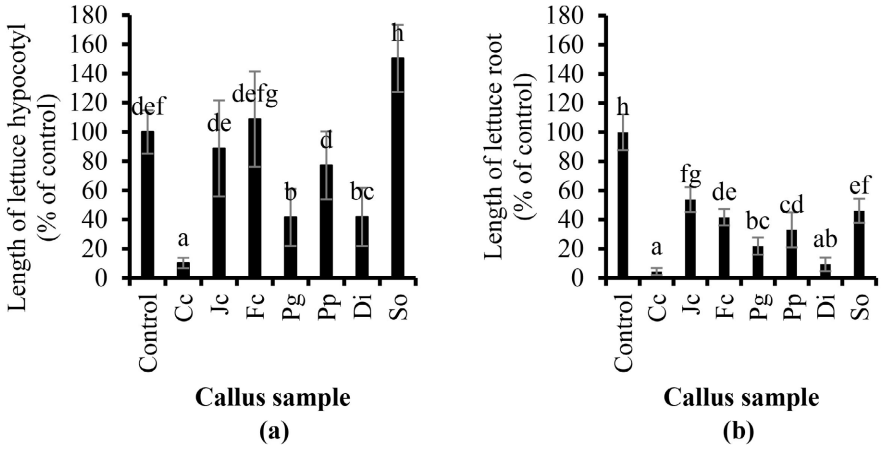

Figure 2. SW bioassay of seven woody plant calli on lettuce hypocotyls (a) and roots (b). Averages with SE (N = 5). Coffee tree (Cc, *C. canephora*), conifer tree (Jc, *Juniperus conferta*), fruit trees (Fc, *Ficus carica* L., Pg, *Punica granatum*, Pp, *Prunus persica*), pongame oil tree (Di, *Derris indica*), and mangrove tree (So, *Sonneratia ovata*).

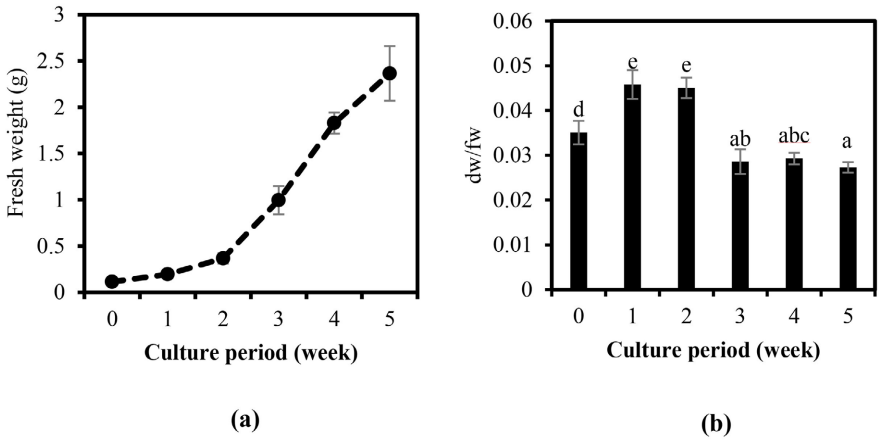

Figure 3. Growth dynamics of coffee nEC, (a) fresh weight per week; (b) dw/fw ratio per week.

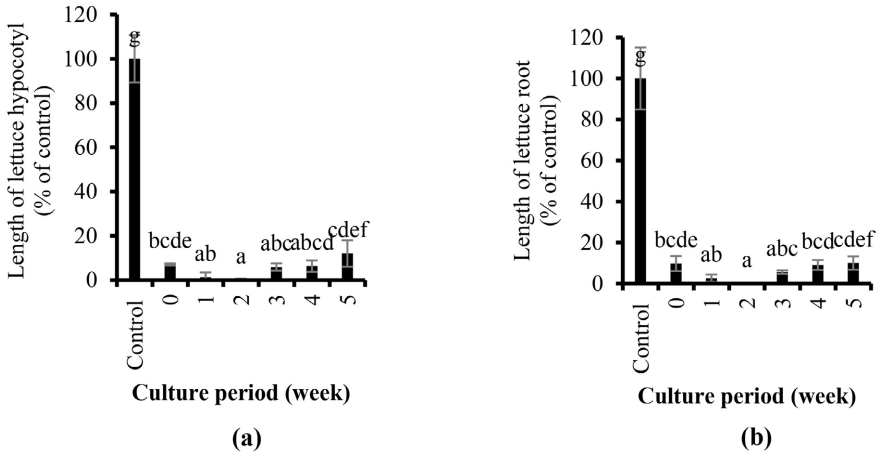

Figure 4. SW bioassay of coffee nEC on lettuce hypocotyls (a) and roots (b) during subculture. Averages with SD (N = 5).

3.3. Allelopathic Activity in Coffee Leaves and Green Beans

The allelopathic activity of coffee leaves (**Figure 5**) did not show any inhibitory effects on the growth of lettuce hypocotyls. Lettuce root growth was slightly inhibited by Ca 10 mg and Cl 50 mg. Stronger inhibition was shown by Cc 50 mg and the strongest was shown by Ca 50 mg, with an inhibition close to 60% of that of the control.

Allelopathic activity in green coffee beans (**Figure 6**) showed a slight inhibition of lettuce hypocotyl growth in the Ca 50 mg and Cc 50 mg samples. The strongest inhibition of the hypocotyl lettuce was close to 50% in the Cl 50 mg samples. Inhibition of lettuce root growth was observed in all samples except for the Cl 10 mg sample. Slight inhibition was shown in a 10 mg Ca sample, and the strongest was shown in a 50 mg Ca sample, with an inhibitory effect nearly 80% of that of the control.

3.4. Profiling of Target Metabolites in Coffee nEC

We measured the levels of the known allelochemicals Tb and Cf in coffee plants as the reference alkaloids. During subculture, there were differences in the Tb and Cf concentrations (**Table 1**) depending on the phase of callus growth. The variation in the Tb/Cf ratio during subculture reflects the conversion efficiency of Tb to Cf in its biosynthetic pathway.

We also identified several distinct unknown peaks suspected to be allelochemicals by profiling the HPLC chromatograms (**Figure 7**). The higher total phenolic content (105 μg/g; **Figure 8**) in the second week also suggested that a key biosynthetic pathway related to the allelopathic activity of coffee calli was activated in the lag phase.

4. Discussions

4.1. Uniqueness of Coffee nEC for Allelopathy Study

Several studies on allelopathic activity in plant cell cultures have already been

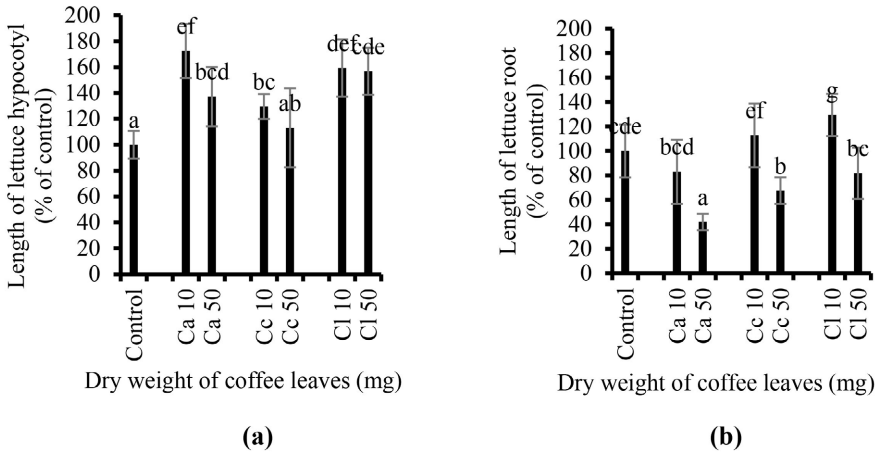

Figure 5. SW bioassay of coffee leaves on lettuce hypocotyls (a) and roots (b). Averages with SE (N = 5). Ca, *C. arabica*; Cc, *C. canephora*; Cl, *C. liberica*.

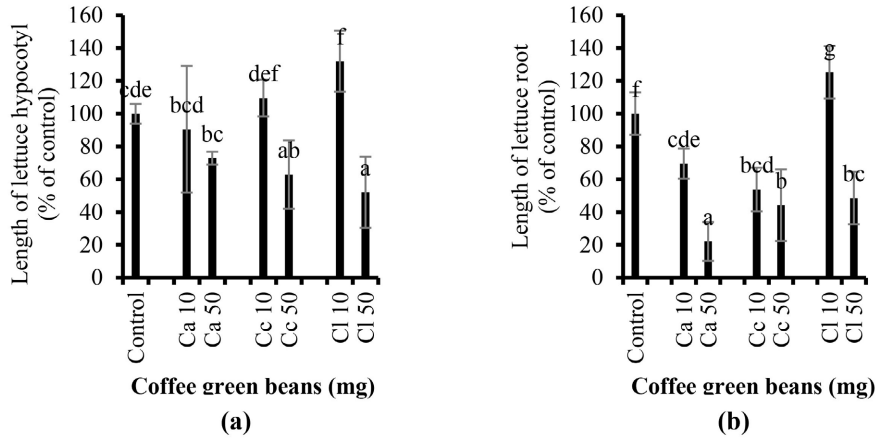

Figure 6. SW bioassay of coffee green beans on lettuce hypocotyls (a) and roots (b). Averages with SE (N = 5). Ca, *C. arabica*; Cc, *C. canephora*; Cl, *C. liberica*.

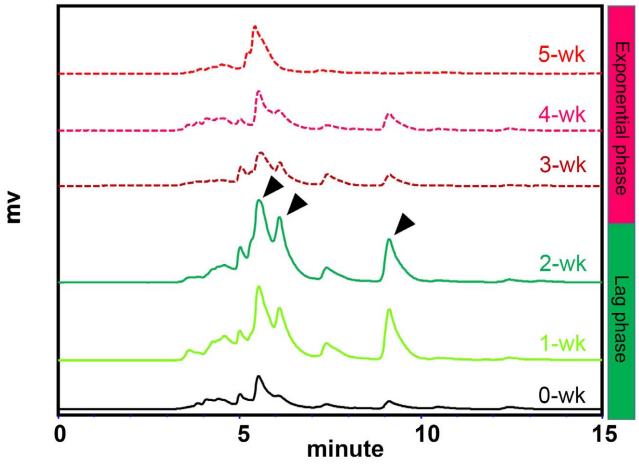

Figure 7. HPLC chromatogram profiles of coffee nEC during subculture. Solid lines indicate chromatograms in lag phase. Dashed lines indicate chromatograms in exponential phase. Arrow heads indicate prominent unknown peaks.

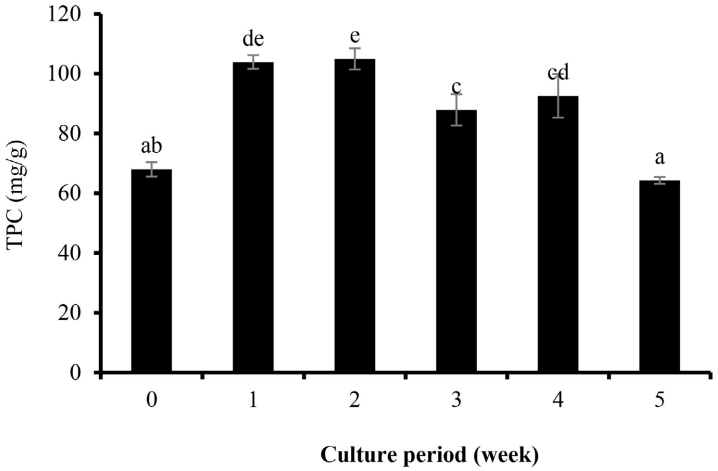

Figure 8. Total phenolic contents by the Folin-Ciocalteu method.

Table 1. Tb and Cf concentration and Tb/Cf ratio in coffee nEC aqueous extracts.

Culture period (week)	Concentration (μM)		Tb/Cf
	Tb	Cf	
0	2.08	4.99	0.42
1	2.47	3.77	0.65
2	1.18	4.09	0.29
3	0.05	1.14	0.04
4	0.17	3.98	0.04
5	0.60	4.14	0.15

published, such as asparagus calli [9], small everlasting calli [8], and suspension cultures of leguminous mangrove plants [10]. Previously, we reported a modified SW-method bioassay for Robusta coffee calli and found that nEC showed strong allelopathic activity [13]. In this study, we compared the allelopathic activities of seven woody plant calli and concluded that coffee nEC had the strongest inhibitory effect on lettuce seedling growth. We then focused on clarifying the cellular growth dynamics of coffee nEC during subculturing. Two typical phases, the lag phase (1 - 2 weeks of culture) and the exponential phase (3 - 5 weeks of culture), were observed, with variations in the dw/fw ratio. The most prominent culture period expected to promote allelopathic potential is the lag phase. In this phase, metabolite mobilization begins and the synthesis of proteins and specific metabolites occurs without cell multiplication [16]. Next, the calli entered the exponential phase with a high proliferation rate reaching a 21-fold increase in weight at five weeks. Cultured cells with high proliferation potential have the potential to create a large source of cell biomass [17]. During this phase, the moisture content increased because the callus actively took up nutrients and water to regulate rapid cell division and growth. The allelopathic activity in this phase also showed a strong inhibitory effect, considering that allelochemicals

were continuously produced.

Allelopathic activity in coffee leaves and green beans was less inhibitory than that in calli. Interestingly, some samples promoted the growth of lettuce seedlings. All leaf samples promoted lettuce hypocotyl growth, likewise for green bean samples Cc 10 and Cl 10. Lettuce root growth was also promoted by leaf samples Cc 10, Cl 10 and bean sample Cl 10.

4.2. Possible Allelochemicals in Coffee nEC

Phytotoxins present in coffee tissues include Cf, Tb, theophylline, paraxanthine, scopoletin, and chlorogenic, ferulic, p-coumaric, p-hydroxybenzoic, caffeic, and vanillic acids [18]. We identified Tb and Cf in coffee nEC using HPLC. Caffeine biosynthesis involves the conversion of xanthosine into Cf [19]. The final step in this biosynthetic pathway is the conversion of Tb to Cf, which involves three successive methylation reactions catalyzed by Cf synthase [21]. The N1, N3, and N7 positiocons of Tb are then methylated, resulting in the formation of Cf [20] [21] [22]. The Tb/Cf ratio is an indicator of the rate of target biosynthesis in cells; a smaller value indicates faster conversion from Tb to Cf. The conversion occurs gradually following proliferation and may accumulate steadily throughout the culture period, reaching its maximum level at a certain stage and then declining [16].

The inhibitory effects of Tb and Cf on lettuce cell proliferation have been reported [23]. For 1 mM concentration, Tb and Cf showed 84% and 100% inhibition, respectively. The Tb and Cf concentrations in coffee nEC were not high (μM order). Several prominent unknown peaks detected in this study suggested the existence of new candidate compounds as allelochemicals. Most allelochemicals are derived from acetate or amino acids that participate in the shikimic acid pathway [24]. The plant shikimic acid/shikimate/phenylpropanoid pathway directs bulk carbon flow toward the biosynthesis of aromatic amino acids (*i.e.*, tyrosine, phenylalanine, and tryptophan) and numerous aromatic phytochemicals [25]. Further analyses are required to determine the unknown peaks.

The TPC results also showed the possibility of the presence of phenolic allelochemicals in coffee nEC. Phenolic compounds are among the most important and common plant allelochemicals found in ecosystems. They are chemical compounds comprising a hydroxyl group (-OH) that bound directly to an aromatic hydrocarbon group. Phenolic allelochemicals have been found to inhibit plant root elongation and cell division, alter cell ultrastructure, and interfere with the normal growth and development of the whole plant [24].

5. Conclusion

In this study, we investigated the relationship between cellular growth dynamics and allelopathic activity was discussed by adapting the SW bioassay, HPLC analysis, and the Folin-Ciocalteu method. We concluded a key biosynthetic pathway in relation to the allelopathic activity of coffee callus will be highly activated in

the lag phase. Most allelochemicals are obtained from acetate or amino acids that participate in the shikimic acid or phenylpropanoid pathway and its derivatives. For future studies, we are constructing detailed analysis for the unknown peaks using LC-QTOFMS method with our prominent coffee cell culture system.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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