Production of the secondary metabolite catechin by in vitro cultures of Camellia sinensis L

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Production of the secondary metabolite catechin by *in vitro* cultures of *Camellia sinensis* L

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Abstract:
Background: Catechin is one of the secondary metabolites in *Camellia sinensis* L. that is alternatively produced through *in vitro* cultures. The *in vitro* culture product is possibly improved by optimizing the culture medium with the addition of growth regulators and precursors. The purpose of this study was to confirm the success of the secondary catechin metabolite production through the *in vitro* culture of *C. sinensis* L in a relatively short time.
Methods: The secondary catechin metabolite product is obtained in about 40 days. The study was conducted by (1) leaf cutting for inoculation in Murashige and Skoog media with 1 μg/mL of 2,4-dichlorophenoxyacetic acid growth regulator; (2) the inoculation of callus multiplication on the same medium as a partially modified inoculation media condition with the addition of 1 μg/mL of 6-benzylaminopurine (BAP) and 2 μg/mL of 2,4-dichlorophenoxyacetic acid at concentration; (3) callus multiplication developed on a new medium containing phenylalanine precursors (300 μg/mL); (4) testing growth by harvesting the callus and weighing the wet weight of its biomass and (5) identification of the callus qualitatively and quantitatively by using high-performance liquid chromatography (HPLC).
Results: The level of secondary catechin metabolite produced was 2.54 μg/mL and 12.13 μg/mL in solid and suspension media, respectively.
Conclusions: It is concluded that the method is effective and efficient in producing catechin product from *C. sinensis* L.

Keywords: *Camellia sinensis* L, catechin, growth regulator, *in vitro* culture, phenylalanine, secondary metabolite
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Introduction

The secondary metabolite catechin is present in various plant cultivars of *Camellia sinensis* L [1], [2]. Catechin and its derivatives are multi-functionally bioactive, including as anticancer [3], [4], [5], in obesity [6], microbially [7], in anti-hepatitis C [8], as phyto-metabolites [9], and as anti-oxidants. As an antioxidant, there are three kinds of mechanisms that bind to hydrogen, prevent oxidation reactions, and free radical acceptors [10]. In the field of food and drink, it acts as a functional food [11]. In the field of agriculture, it can act as a bactericide [12], as a pesticide [13] and as chemical alloys [14]. Catechin, in addition to occurring in the plant, can also be produced through the *in vitro* culture method.

In vitro culture is a potential technique for producing secondary metabolites. There are advantages of *in vitro* culture techniques such as it needs less field for growing the biological source,the production of metabolites may be increased to industrial scale, and the harvesting is performed in a relatively short time. However, the amount of product produced by *in vitro* cultures is usually low when compared to plant-derived extract. Phenylalanine precursors are applied to the cultured callus and suspension to enhance the production [15]. The purpose of this study was to clarify the success of the production of catechin secondary metabolites through the *in vitro* culture method using *C. sinensis* L in a relatively short time.

- **Sutini** is the corresponding author.

Experimental

Callus inoculation

The leaves of the *C. sinensis* L plant were obtained from plants that had been maintained for some time in the greenhouse. The leaves were washed in running water for 15 min [1], [2], [3], [4]. The leaves were immersed in 4% fungicide solution [16] and 1% benomyl bactericide [17] prior to rinsing with sterile water. The leaves were soaked in a 15% sodium hypochlorite solution for 10 min [18] and then rinsed. The leaves were soaked in 70% alcohol solution and 0.1% mercury(II) chloride ($HgCl_2$) for 10 min [18] and then rinsed resulting in explants. These explants were cut an area of about 1–2 cm in a laminar air flow (LAF) cabinet (ESCO, PA, USA). The cut explant was planted/inoculated in a culture chamber containing Murashige and Skoog media modification compositions [19] with 1 µg/mL of 2,1 dichlorophenoxyacetic acid [20]. The explant was deposited in an incubation room for 4–8 weeks to gain the callus.

Callus multiplication

The callus were propagated on Murashige and Skoog media supplemented with 1 µg/mL of 6-benzylaminopurine (BAP) growth regimens [21], [22] and 2 µg/mL of 2,4-dichlorophenoxyacetic acid [23] to multiply the callus biomass.

Treatment with phenylalanine precursors in *in vitro* culture of callus and suspension

Phenylalanine precursors (300 µg/mL) was used in the treatment for *in vitro* culture of callus and suspension [24], [25]. Three hundred milligrams of phenylalanine were dissolved in 1000 mL of distilled water and then filtered with Millipore filter paper. The treatment is done in two stages. The first stage was to create a new media composition similar to the callus multiplication medium with each bottle added with 300 mg/L phenylalanine. In this stage, 100 mg of callus was inserted into a vial containing callus culture medium and incubated for 30 days. The second stage was to make the suspension solution media of Murashige and Skoog media without agar. Phenylalanine (300 µg/mL) was added. In the second stage, a 100 mg copped callus was inserted into the suspension culture. The culture was then shaken at a speed of 120 rpm with 500 lux lighting for 30 days [26].

Examination of growth of callus culture and suspension

A sub culture was done every 6 days. Callus growth was examined every 6 days for 30 days [27]. Callus growth was measured by examining the changes in the wet weight of the callus. The examination of growth in the suspension media was measured by examining the changes in the wet weight of the callus suspension.

Extraction

Biomass of *in vitro* culture callus and the suspension was weighed to 500 mg and then smoothed, after finely being dissolved with 5 mL of methanol 80% [28], [29], and macerated for 1 day. This maceration was repeated 3 times. The filtrate was separated by residue and stored in closed bottles. Concentrated HCl was added to the extract (by as much as two drops) and then heated to boiling for 10 min. After that the cold extract was concentrated up to 3 mL using nitrogen gas. The concentrated extract was again extracted using 5 mL of ethyl acetate [30] this was repeated 3 times for analysis using high-performance liquid chromatography (HPLC) [31].

Catechin analysis with HPLC

Biomass extract of the callus culture and suspension was obtained using methanol extract [32]. The obtained solution was then filtered using Whatman filter paper with a pore diameter of 0.2 µm, it was then injected into HPLC-ultaviolet (UV) using as much as 20 µL, and then eluted with a mobile phase containing 1% acetic acid and 100% acetonitrile [33]. The chromatogram profiles were then obtained and compared to confirm the existance of the secondary cathecin metabolite.

Results

The present study showed that callus was formed with a yellowish-green color and a compact structure after 25 days. Callus formed as bulging leaf explants. The next step of the observation showed a groove form of callus. Subsequently, partial callus appeared as the next morphological feature. It was shown that the complete morphology changes as callus was formed. The present study showed the color of clear yellowish-green callus. In further observation, the color turned to brown (Table 1, Figure 1).

Table 1: Appearance profile of callus forming.

Culture duration	Color	Texture
0	Green	Cutting leaves
10	Green	Bulging
15	Green	Indentation
25	Pale green	Callus on edge
35	Domination green to yellowish	Full callus

The callus of *C. sinensis* L plant were observed visually at 0-, 10-, 15-, 25- and 35-day culture age. The observation includes color change and texture in every stage.

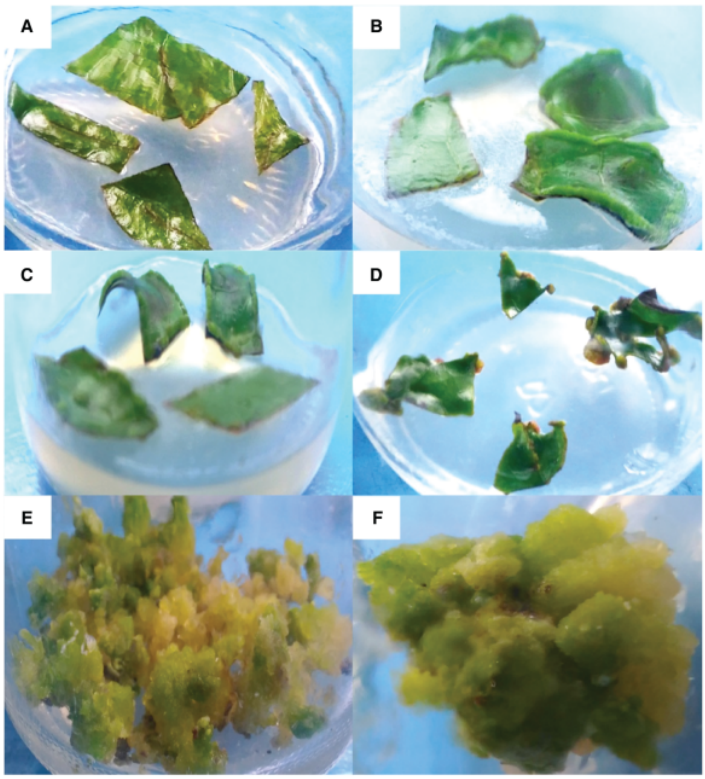

Figure 1: The representatives photograph of callus development from *C. sinensis* L plant.
The feature changes were observed in culture day 0 (A), 10 (B), 15 (C), 25 (D), 35 (E, F). (E) and (F) represent the proliferated callus of *C. sinensis* L plant.

The present study demonstrated that the production of callus biomass with the use of solid media (Murashige and Skoog) added with growth regulator 2,4-dichlorophenoxyacetic acid, BAP and phenylalanine precursor, increased the callus wet weight. The results showed that the production of callus biomass in solid medium hit the maximum amount at around 825 mg wet weight in 40 days. The growth slightly increased at

day 10 and was gradually augmented in the subsequent 30 days (Figure 2). However, the production increased over 40 days to 900 mg wet weight. Moreover, the growth increased steeply in the 10th day after inoculation (Figure 3).

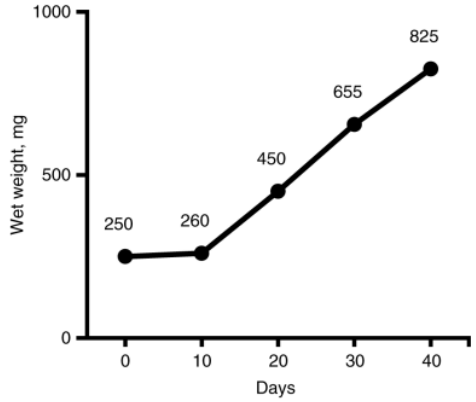

Figure 2: Wet weight of callus biomass with solid medium.
The data were obtained from 40 days of experiments.

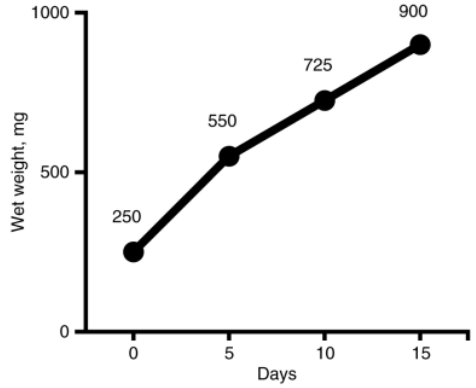

Figure 3: Wet weight of callus biomass with suspension medium.
The data were obtained on day 0, 5, 10 and 15.

The callus extract of a 35-day solid medium and a 15-day suspension medium were analyzed using HPLC resulting in the catechin retention time and peak area (Table 2). Levels of catechin from callus extract on solid media and suspension media were, respectively, 2.54 µg/mL and 12.13 µg/mL.

Table 2: The result of liquid chromatography profiling of cathecin of callus extract in different medium.

Medium	Retention time	Area
Solid medium	13.561	68.08
Standard	13.756	1668.21
Suspensed medium	13.619	404.92

Disscussion

Callus formation using the *in vitro* culture technique begins with leaf inoculation of *C. sinensis* L on Murashige and Skoog media with 1 µg/mL growth regulator (2,4-dichlorophenoxyacetic). Twenty-five days after inoculation, a callus was formed with a yellowish-green color and a compact structure. It was demonstrated that

the phases of callus formation were positively associated with the formation of the explants. Callus formed as bulging leaf explants. The subsequent phase exhibited a groove form. In the next phase, partial callus appeared and was followed by complete morphology changes into a callus (Table 1, Figure 1). This change in morphological appearance is relevant to the previous report in the *Aglaonema* sp. plant [34]. The result of the present study revealed the color of clear yellowish-green callus indicating the young and healthy cell conditions (Table 1, Figure 1). It is known that the callus produced is commonly used for various purposes in industrial agriculture, the health industry, and the food and beverage industry. The healthy cells are usually maintained for more than 15 years for germplasm supply. It is reported that Murashige and Skoog media with 2,4-dichlorophenoxyacetic acid growth regulator as was used in the present study is generally used for callus proliferation [35]. As the age of callus increases, the color of the cells turns to brown. It is important to immediately transfer the culture into the new media for preservation. The present study showed the feature of the compact callus structure that is a readily reproducible callus (Table 1, Figure 1).

Murashige and Skoog media reportedly increases callus multiplication and produces more biomass. This agrees with a previous study stating that Murashige and Skoog medium often provides a good result when used in *in vitro* study [36]. The acceleration of the proliferation and callus biomass production were obtained by combining the addition of BAP growth regulator (1 μg/mL) and 2,4-dichlorophenoxyacetic acid (2 μg/mL) (Figure 2). To obtain the proliferation and biomass of this callus, the concentration of zinc pyrithione (ZPT) was optimized first. Optimizing the ZPT concentration is important to see the response of each plant or explant from different plant organs. The difference response is related to the presence of endogenous ZPT present in each plant explants of mutual synergy or antagonist [37]. The present method is relevant to the previous study showing the use of BAP (0.1 μg/mL) in the plant culture of *Justicia gendarussa*. Incubation for a week regenerates a pale green callus into a pigmented one. Increasing the BAP level to 2 μg/mL changes the callus into an embryonic callus [38], [39]. The use of BAP and Murashige and Skoog media in *Scutellaria altissima* produces callus containing the secondary metabolites baicalin, wogonoside, and verbascoside [40].

The present study demonstrated that the production of callus biomass with the use of solid media (Murashige and Skoog) added with a growth regulator 2,4-dichlorophenoxyacetic acid, BAP, and phenylalanine precursor increased the callus wet weight (Figure 2). This study is relevant to the previous study showing that the use of a BAP growth regulator in the same concentration successfully produced a callus [41]. The use of phenylalanine reportedly affects the formation of some secondary metabolites. In connection with the present study, phenylalanine is a substrate for catechin formation in the phenylpropanoid pathway [42]. Thus, the present study suggests the effectiveness of modified solid media to produce the callus biomass from *C. sinensis* L culture.

Callus biomass with the use of liquid medium (Murashige and Skoog) modified with the addition of growth regulators 2,4-dichlorophenoxyacetic acid (1 μg/mL), BAP (1μg/mL) and 300 mg/L phenylalanine precursors. It is revealed that the biomass was able to be properly harvested within 15 days (Figure 3). Our previous study shows that biomass is harvested after 3 weeks [43]. Other previous studies showed that phenylalanine is a pre-substance involved in the phenylpropanoid biosynthesis pathway for the formation of catechin secondary metabolites [44], [45]. Taken all together, it is suggested that the suspension media may also generate an effective production of callus biomass.

The callus extract of a 35-day solid medium and a 15-day suspension medium were analyzed using HPLC resulting in the catechin retention time and peak area (Table 2). The catechin level was theoretically obtained by multiplying the ratio of the callus extract peak area and the standard peak area by the standard concentration used in the experiment. Levels of catechin from the callus extract on solid media and suspension media were 2.54 μg/mL and 12.13 μg/mL, respectively. It is known that the issue of the stability of catechin might influence the level analysis. Thus, further research is needed to clarify the exact concentration of catechin in the cultures of *C. sinensis* L. Catechin is a derivative of flavonoids whose existence is derived from the biosynthesis of phenylpropanoid pathway combining phenylalanine and malonyl-CoA [46]. This catechin is also found in *in vitro* culture of the *Arbutus andrachne* L plant [20]. Recent studies using sucrose *on in vitro* cultures in the rootstock of plant *C. sinensis* explants markedly increase the amount of catechin [47]. Further research is needed to clarify the best conditions in which the catechin metabolite production may be enhanced.

Conclusion

Our result indicates that the developed method is an effective method as the production of catechin secondary metabolite biomass is accomplished in only about 20 days. Besides the effectiveness of the presently introduced method for *C. sinensis* L, it is suggested that the method exhibits a high productivity demonstrated by more than a 100% increase in the production in 40 days.

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