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The Effect of Callus Age on Biomass and Flavonoid Production in *Talinum paniculatum* In Vitro Culture

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Abstract

Talinum paniculatum (Javanese Ginseng) is an herbaceous plant belonging to Talinaceae family that has been known as an important medicinal plant. Flavonoid is one of the prominent bioactive compounds that are contained in all parts of T. paniculatum and has been known for its several bioactivities, including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and anti-cancer. Callus culture can be used to ensure the quantity and quality of flavonoid production. Callus age is one of the important factors that needs to be considered in order to produce optimum biomass and flavonoid production. Therefore, this research aimed to investigate the effect of callus age on biomass and flavonoid production in T. panicultaum in vitro culture. Young leaves were used as an explant, and callus induction was performed using MS solid media supplemented with kinetin 3 mg/L and 2.4-D 2 mg/L. Biomass and flavonoid production were observed during 0, 28, 35, 42, and 49 days. Results showed that biomass and flavonoid production were influenced by callus age. The production of flavonoid in T. panicultaum callus culture was not linear with the growth of callus (biomass), and the optimum flavonoid production resulted in the 28th days of callus age (49.100 mg QE/g), while the optimum biomass resulted in the 49th days of callus age (4.295 g). It can be concluded that the optimum conditions for biomass and flavonoid production in T. panicultaum callus culture are influenced by different callus ages

Keywords: *Biomass, Bioactive compounds, Callus, Flavonoid production, Talinum paniculatum*

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INTRODUCTION

Talinum paniculatum, also known as Javanese Ginseng or Som Jawa, is one of the important medicinal plants. This herbaceous plant is a member of the Talinaceae family which has a similar root morphology to Korean *Panax ginseng* (Lakitan et al., 2021). *T.paniculatum* has known for several important bioactive compounds such as saponin, flavonoid, tannin, and steroid (Aini & Susilo, 2023). This plant has several pharmacological effects including antioxidant (Lestario dkk., 2009), anti-cancer (Liu et al., 2018), anti-diabetic (Jenie & Indraswari, 2021), anti-microbial (Cerdeira et al., 2020), to treat cardiovascular and urinary tract diseases (Tolouei et al., 2021) and to enhance spermatozoa motility and viability (Aini & Susilo, 2023). Flavonoid is one of the prominent bioactive compounds that are contained in all parts of *T.paniculatum* and has been known to its several bioactivities, including antioxidant, anti-inflammatory, antimicrobial, antidiabetic and anti-cancer (Eddijanto et al., 2022).

The increasing demand for bioactive compounds such as flavonoid in the pharmaceutical industry needs a major breakthrough in efficient supply. Bioactive compounds can be naturally extracted from grown-field plant organs. However, the quantity and quality of bioactive compounds are not sustainable due to dynamic changes of geographic and environmental conditions (Efferth, 2019; Chandran et al., 2020). This, however, has become a major challenge in fulfilling the market demand for sustainable bioactive compounds. In vitro culture can be employed for the production of bioactive compounds, one of which is callus culture. Callus culture has several advantages over traditional whole plant bioactive compounds extraction such as: (1) callus can be easily grown from explant; (2) the selected plant bioactive compounds can be produced irrespective of external factors such as soil composition or climate; (3) cultured cells are not susceptible to attacks from microorganisms or insects; and (4) callus from any plant organs can be easily preserved to produce their secondary metabolites (Efferth, 2019).

Production of bioactive compounds using in vitro culture consisted of two steps: (1) biomass production and (2) secondary metabolites production (Chandran et al., 2020). According to Ramirez-Estrada et al. (2016), the enhancing strategy of secondary metabolites production by in vitro culture should consider the growth phase of callus culture, either in exponential or stationary growth. This growth phase is correlated with the callus age. Previous studies have reported that 8th weeks of *Taraxacum officinale* during exponential growth produced higher triterpene yield than stationary phase (Martínez et al., 2023). Furthermore, flavonoid production from *Stelechocarpus burahol* cell suspension culture showed higher yield during the cell growth in the log phase (Habibah et al., 2017). Based on our best knowledge, there is no information regarding the effect of callus age (growth phase) on flavonoid production in *Talinum paniculatum* in vitro culture. Based on the description above, callus age is one of the important factors that needs to be investigated in order to produce the optimum yield of bioactive compounds through in vitro culture. Thus, to produce the optimum condition for flavonoid content, we analyzed the effect of callus age (growth phase) on biomass and flavonoid production in *Talinum paniculatum* in vitro culture.

METHOD

Research Design

This research used a completely randomized design consisting of 4 treatments (callus age) with 3 replications. The research consists of two steps: callus induction (biomass production) and flavonoid production.

Callus Induction and Biomass Measurement

The leaf explants used in this research were obtained from the Biotechnology Laboratory, Faculty of Biotechnology, Universitas Kristen Duta Wacana, Yogayakarta. The second and third leaves from the shoot apical were used as explants. Healthy and green leaf explants were subsequently washed using a solution of liquid detergent and three drops of Tween 80, then rinsed using running water until clean. Sterilization of leaf explants was performed inside the laminar air flow. Clean leaves were immersed in 70% alcohol for three minutes and washed using sterile aquadest three times. Steriled leaf explants were cut into 1 x 1 cm^2 in size, passing the leaf vein. Explants were inoculated in a solid media of MS (Murashige & Skoog, 1962) supplemented with kinetin (3 mg/L) and 2.4-D (2 mg/L) (Restiani et al., 2022). Biomass and flavonoid production were observed during 0, 28, 35, 42, and 49 days. Callus biomass was measured based on the dry weight of the callus. The dry weight of the callus resulted from the callus being dried in an oven at 40°C until it reached a constant weight.

Callus Extraction and Total Flavonoid Content Analysis

Extraction of callus was carried out based on Manuhara et al.(2015) by drying the callus using an oven at 40°C until it reaches a constant weight. The dried callus was pulverized using a mortar and pestle or a blender. Callus extraction was carried out by maceration method using 96% methanol solvent with a ratio of 1:100 (w/v) for 2 days. The callus powder was macerated and incubated for 24 hours, then the extract was filtered using filter paper. Then, the callus powder powder was re-macerated in the same way. The resulting filtrate obtained was evaporated using a waterbath with a temperature of 65°C until the final total volume is ± 1 mL. Flavonoid production was measured using Total flavonoid content (TFC) based on a modified colorimetric test method from (Park et al., 2020). A total of 100µL (0.1 mL) of extract was dissolved in methanol to a total volume of 1 mL. The solution was mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution was added and incubated for 5 minutes. After that, 0.3 mL of 10% $AlCl_3$ solution was added and allowed to stand for 6 minutes at room temperature. Then, 2 mL of 1 mol/L NaOH solution was added to the mixture and distilled water was added until the final volume of the mixture was right. Distilled water was added until the final volume of the mixture was exactly 10 mL. The mixed solution was allowed to stand for 15 minutes. The absorbance was measured using spectrophotometric method with a wavelength of 510 nm. TFC was calculated from the standard curve plotted

using quercetin as standard with a concentration of 50 - 500 mg/L (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg/L). TFC was expressed in quercetin equivalent (QE)/g dry weight.

Data Analysis

Data on callus morphology (texture and color), biomass (callus dry weight), and total flavonoid content (TFC) were analyzed descriptively. All data were presented as mean ± standard error from three replications.

RESULT AND DISCUSSION

Callus Morphology of *Talinum paniculatum*

T. paniculatum leaf explants cultured in MS medium with the addition of a combination of 3 mg/L kinetin and 2 mg/L 2.4-D showed callus formation (Figure 1). Callus produced from MS solid media and combination of plant growth regulator (2.4-D and kinetin) resulted in callus initiation of 100% (data not shown). The combination of kinetin and 2.4-D is the optimal PGR combination for *T. paniculatum* callus production (Natasha & Restiani, 2019; Restiani et al., 2022). The combination of auxin and cytokinin plays an important role in inducing callus formation and regulating explant regeneration through in vitro culture because these two hormones work synergistically to stimulate cell division, enlargement and differentiation (Habibah et al., 2016). Kinetin from the cytokinin class plays a role in triggering cell division and differentiation, while the addition of auxins such as 2.4-D can stimulate cell division and enlargement so as to stimulate callus formation and growth.

Callus began to form on 4 days after inoculation (DAI) (Figure 1b). Callus began to swell on 2 DAI (Figure 1a). This swelling is caused by leaf explants absorbing water and nutrients from the medium. Water absorption is also caused by an increase in exogenous auxin hormone (2.4-D) and endogenous auxin hormone in the explant tissue which triggers the breakdown of cellulose that makes up the cell wall because the activity of the expansin protein is activated so that the loose cell wall triggers water absorption into the cell and the explants begin to enlarge. On 4 DAI, callus began to form at the edge of the wound site and at the tip of the leaf bone. This is because callus is formed as a natural response to wound closure triggered by the wounding signal, auxin and cytokinin hormones and nutrients present in the media (Ikeuchi et al., 2013; Habibah et al., 2016). Cell division of callus began to occur rapidly after 14 DAI (Figure 1d-f). This phase showed that the cell division phase occurs exponentially (exponential phase). The time of callus initiation in *T. paniculatum* leaf explants is relatively fast (4 DAI). This is because *T. paniculatum* is an herbaceous plant, where this type of plant is genotypically relatively easier to propagate through in vitro culture. In addition, explants derived from leaves are one type of explant that is more responsive to active cell division because leaves consist of parenchyma tissue which is naturally meristematic (Bhojwani & Dantu, 2013).

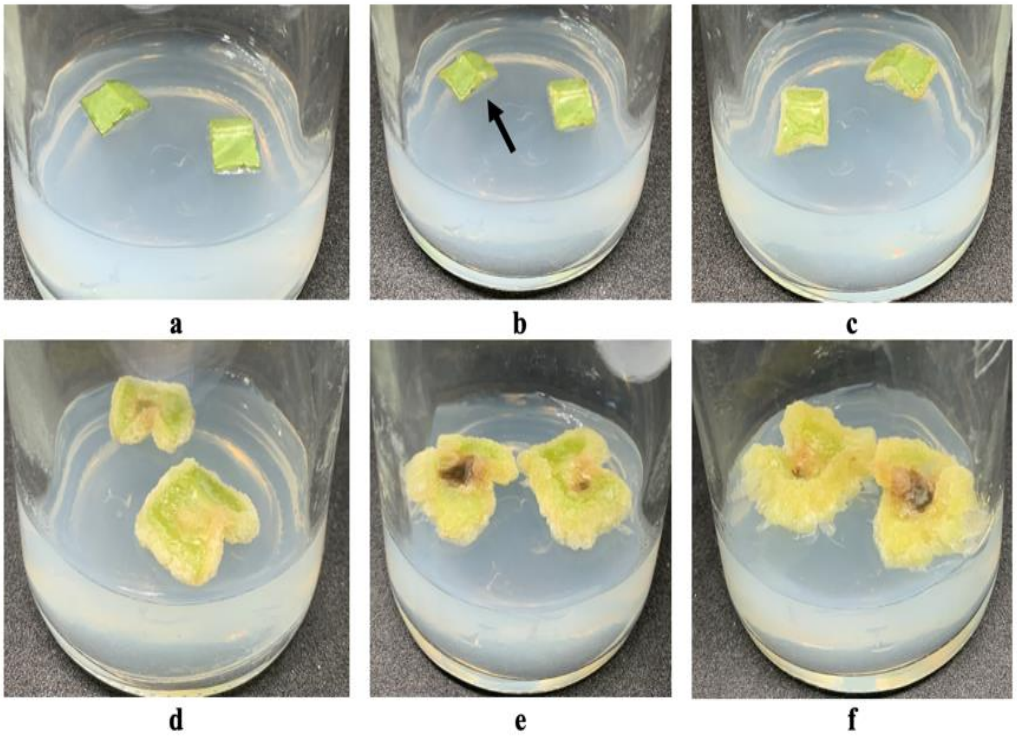

Figure 1. Callus development stages from *Talinum paniculatum* leaves explants (a) 2 DAI (b) 4DAI (c) 7 DAI (d) 28 DAI (e) 35 DAI and (f) 42 DAI
(Note: Black arrow showed initiation step of callus)

T. paniculatum callus produced in this research is a callus with a friable structure in green yellowish color. Friable callus is ideal for biomass propagation through cell suspension culture. The purpose of callus production in this research is to produce secondary metabolite compounds, while the ideal callus for secondary metabolite production is callus with a compact structure. According to Habibah et al. (2023), the compact callus texture is characterized by a dense arrangement of cells, the form of a solid bulge, and less water content, which contributes to its firm structure. The difference in callus texture has a different ability to produce metabolite compounds. Based on the research of Sugiyarto & Kuswandi (2014), callus with compact texture has greater potential in producing secondary metabolites than friable callus texture because compact texture has more metabolite accumulation. *T. paniculatum* produces various kinds of secondary metabolites such as flavonoid, alkaloid, saponin, tannin, and sterol (Silalahi, 2022). However, in this research, the callus produced had a friable texture which might be caused several factors such as the type of explant, the type of PGR added to the media, and the growth phase of the callus.

The Effect of Callus Age on Biomass of *Talinum paniculatum*

This research aims to determine the effect of callus age on increasing callus biomass and total flavonoid production from *T.paniculatum* callus. Callus biomass used in this research is the dry weight (DW). Biomass is one indicator of callus growth. Increasing biomass is one of the important factors in the production of secondary metabolites through in vitro culture. Based on the results in Figure 2, the longer the age of the callus (49 days) resulted in the increase in biomass (4.295 ± 0.009) g compared to the dry weight of the callus at the age of 28 days (0.707 ± 0.200 g). This result was in line with the increase in biomass of *Sophora flavescens* callus cultured for 8 weeks which increased (15.4025 g) compared to 2 weeks (6.725 g) and 4weeks 4 (10.4 g) (Park et al., 2020). The increase in biomass along with the age of the callus is due to the activity of division and enlargement of the cells that form the callus. The interaction of exogenous hormones 2.4-D (auxin) 2 mg/L and kinetin (cytokinin) 3 mg/L added to MS medium with endogenous hormones present in leaves explants causes an increase in callus growth activity. 2.4-D is one type of hormone from the auxin group that plays a role in stimulating callus initiation and proliferation. Kinetin from the cytokinin group also plays an important role in supporting the increase in callus biomass because it regulates cellular processes during growth and development, namely cell division. In addition, the absorption of nutrients and water from the culture medium into cells also helps growth activity and directly affects the increase in biomass (Indriani et al., 2020; Park et al., 2020).

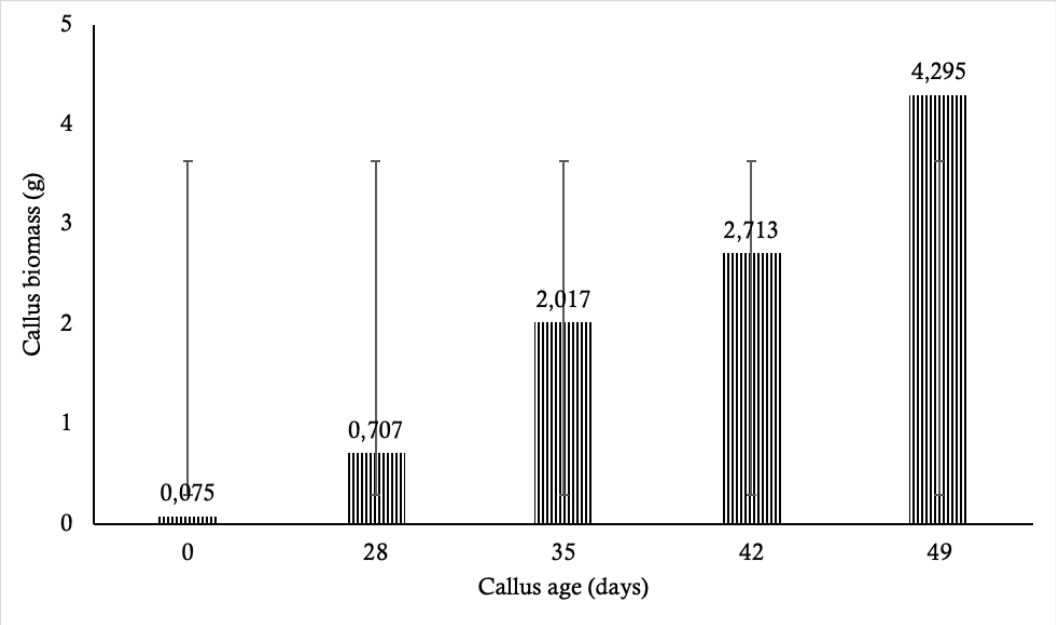

Figure 2. Callus biomass at different age of callus of *Talinum paniculatum*

The Effect of Callus Age on Flavonoid of *Talinum paniculatum*

In addition to affecting the increase in callus biomass, callus age showed its influence on the production of total flavonoids from *T. paniculatum* callus. Based on the results in Figure 3, the increase in callus age (49 days) caused a decrease in the total flavonoid content of *T. paniculatum* callus (15.590 mg QE/g) compared to the total flavonoid content of callus on day 28th which amounted to 49.100 mg QE/g. This is in line with the research of Tan et al.(2010), which reported the optimal flavonoid content of *Centella asiatica* callus on 12 DAI (5.26 ± 0.09 mg/g), which is during callus at the early exponential phase, while after 12 DAI, the total flavonoid content become declining until the stationary phase. This is might be in the exponential phase, the callus is actively dividing and increasing biomass so that in this phase the flavonoids synthesized are also higher (Tan et al., 2010). Furthermore, Habibah et al. (2017) also reported that flavonoid production from *Stelechocarpus burahol* cell suspension culture showed higher yield during the cell growth in the log phase than stationary phase. This research also reported that the production of flavonoids in the cell suspension cultures of *S. burahol* was not in line with its growth. The results of this research are not in line to the results of Park et al. (2020) who reported an increase in total flavonoid content occurred in callus that had a larger biomass (8 weeks) compared to the callus at 4 week (during the exponential phase).

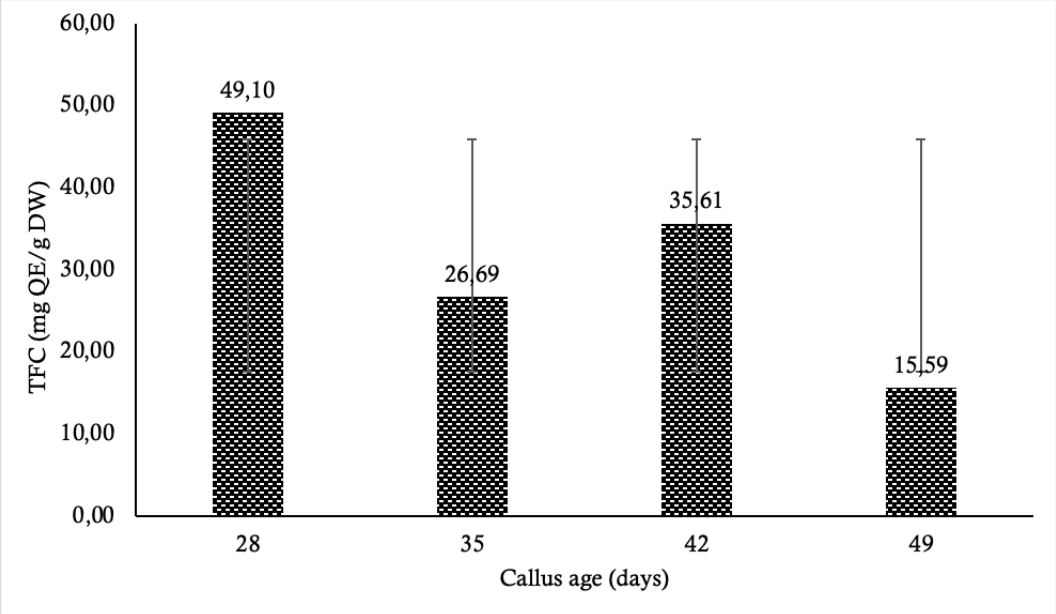

Figure 3. Flavonoid production at different age of callus of *Talinum paniculatum*

In general, secondary metabolite compounds in in vitro cultures are produced when cells enter the stationary phase, where at this stage cell division and enlargement activities have decreased so that cells continue the secondary metabolic stage. In addition, nutrients that begin to run out at the stationary stage are also a factor for the initiation of secondary metabolite synthesis as a result of nutrient stress experienced by callus cells. However, the results obtained in this research show that

flavonoid synthesis actually takes place when the callus cells are actively grown so this indicates that flavonoids are one of the secondary metabolites that can be produced in the exponential growth phase. The 28-days callus age will be used in determining the optimal sucrose concentration and PGR combination for increasing biomass and callus production of *T. paniculatum.*

CONCLUSION

The present research demonstrated that biomass and flavonoid production in *Talinum paniculatum* in vitro culture were influenced by the callus age. The production of flavonoid in *T. paniculatum* callus culture was not linear with the growth of callus (biomass). The optimum flavonoid production resulted in the 28th days of callus age (49.100 mg QE/g), while the optimum biomass resulted in the 49th days of callus age (4.295 g).

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Conflict of interest : The authors declare that they have no conflicts of interest.

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