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Influence of Combination of Sucrose Concentration and Immersion Frequency on Biomass and Flavonoid Production of *Gynura procumbens* (Lour.) Merr Callus Culture in Temporary Immersion Bioreactor

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Original Research Article

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Abstract: The concentration of sucrose, in many cases could influence the production of biomass and secondary metabolite because sucrose is the main carbon source of metabolism, while liquid culture method has many advantages but has problems such as asphyxia and hyperhydricity because of long immersion. Temporary immersion bioreactor could solve problems by way of the immersion frequency. The study aimed to investigate the influence of combination sucrose concentration and immersion frequency on biomass and flavonoid production of *Gynura procumbens* in temporary immersion bioreactor. Callus was obtained from stem nodes and stem internodes that were grown in MS agar medium supplemented with 0.1 mg L^{-1} 2.4-D (2.4-dichlorofenoxyaceticacid) and 0.1 mg L^{-1} IAA (indole acetic acid). In temporary immersion bioreactor (modification of RITA) calli were cultured in MS liquid medium supplemented with various concentration of sucrose (3%, 5%, and 7%) and immersion frequency of 15 min at 12 h interval and 5 min at 3 h interval. Cultures were maintained for 28 days. Results showed that combination of sucrose 5% and immersion frequency of 15 min at 12 h interval produced the highest biomass, while the combination of sucrose 7% and immersion frequency of 15 min at 12 h interval produced the highest flavonoid.
Keywords: *Gynura procumbens*, temporary immersion bioreactor, biomass, flavonoid, callus culture.

INTRODUCTION

G. procumbens is a plant of the Asteraceae family that is important and widely used in Southeast Asia, especially in Indonesia, Malaysia and Thailand [1,2]. This plant is used as a medication to treat fever, rash, kidney disease, migraine, constipation, diabetes mellitus, hypertension, and cancer [3].

G. procumbens has several bioactive compounds such as flavonoids, saponins, alkaloids, tannins, terpenoids and sterol glycosides [4]. Flavonoids have many health benefits including antioxidant, anti-inflammatory, anticancer, anti-cardiovascular, antiaging, antistroke and antiasthma [5-11]. Based on the huge benefits of flavonoids, it is necessary to develop the production of flavonoids on a large scale. The production can be increased by tissue culture techniques.

Tissue culture is a technique for producing plants or secondary metabolites on a large scale.Some of the advantages of tissue culture techniques include certain compounds can be produced under controlled conditions; it is more easily reproduced; the products are consistent both quality and quantity; it can manipulate to form new compounds that are not found in the mother plant; and when producing the product do not depend on environmental conditions such as geography, climate and season. Flavonoid compounds can be enhanced by manipulating the environment, such as the lighting period and the addition of supplement in the medium [12]. The application of callus culture techniques use by leaf explants was able to provide the demand for in-vitro production of secondary metabolites of flavonoids [13]. In several reports, callus cultures were able to increase callus biomass and flavonoid content [14].

Several studies have also shown that the genes that encode enzymes involved in the phenylpropanoid pathway can be influenced by carbohydrate sources, such as glucose and sucrose which act as signal molecules in the biosynthetic pathway. Sucrose is also an important carbon source for secondary metabolic formation [15]. Sucrose does not only supply carbon

skeleton and secondary metabolites, but also regulates signals and molecules [16].

Culture system using a liquid medium can provide more uniform culture conditions, can facilitate the replacement of the medium, the direct contact between explants and medium, and can use a place that is larger than the solid medium. However, liquid culture techniques have several disadvantages such as cells experience a lack of oxygen, can damage cells, cause vitrification [17] and hyperhidrisity [18]. The problem can be overcome through the development of a liquid culture method in the bioreactor, namely, the Temporary Immersion System (TIS). Temporary immersion bioreactor is a type of agitated bioreactors. Several studies have used liquid culture methods in temporary immersion bioreactors such as increasing the efficiency of somatic embryo formation of palm (*Bactris gasipaes* Kunth) [17, 19] and regenerating of the somatic embryo in *Elaeis guineensis* [20] using RITA$^{@}$bioreactor.

Studies on the production of biomass and flavonoid content in *G. procumbens* have been conducted in various cultures such as adventitious root culture [21-23], shoot culture [24] and callus culture in solid media to produce secondary metabolites [14]. However, there have been no studies in using a temporary immersion bioreactor to increase the biomass and flavonoid production of *G. procumbens* callus.

MATERIALS AND METHODS

Materials

Gynura procumbens (Lours.) Merr was obtained from the Botanical Garden Purwodadi, Pasuruan, East Java, Indonesia. The explants used were nodes and internodes stem based on the optimization of the ability of the sources of explants (leaves, stem nodes, internodes, and petioles) in inducing callus formation.

Callus Induction

G. procumbens stems cleaned using liquid detergent and rinsed using tap water. Then the stem was sterilized using 10% Clorox (v/v) for 5 mins and rinsed 3 times with sterile distilled water. The stems were drained on sterile filter paper and cut into the nodes and internodes with a size of ± 0.5 cm. Explants were grown in MS solid medium supplemented with 0.1 mg/L 2,4-D and 0,1 mg/L IAA. The explants were maintained for 28 days at a temperature of 25 ± 3°C in light conditions (white fluorescence lamp 20 Watt).

Callus culture in Temporary Immersion Bioreactor

A 5 g of callus from stem nodes and internodes explants inductions were used as explants for culture in Temporary Immersion Bioreactor (TIB). The TIB design was modified according to the RITA® bioreactor system with a medium volume (working volume) of 250 mL. Callus was cultivated in TIB with a combination treatment of sucrose concentration (3%, 5%, 7%) and variations in immersion frequency (15 min at 12 h interval and 5 min at 3 h interval). The liquid medium used was MS medium supplemented with 0.1 mg/L 2.4-D and 0.1mg/L IAA. The medium pH was adjusted at 5.8. Cultures were kept for 4 weeks at a temperature of 25 ± 3^{o}C in light conditions (white fluorescence lamp 20 Watt).

Extraction and Analysis of Flavonoid Content

A 0.5 g dried biomass powders of each callus treatments were soaked with 10 ml of methanol at room temperature for 24 hours. This procedure was done twice. The extracts were filtered on the filter paper and then concentrated to 6 ml at room temperature. The methanol extracts were partitioned with n-hexane with a ratio of 1: 1 to remove non-polar compounds. Afterward, the methanol extracts were partitioned with ethyl acetate with a ratio of 1: 1 [25]. The extracts of ethyl acetate were then analyzed the content of the flavonoid.

The analysis of total flavonoids in methanol extract was determined by spectrophotometer. A 0.25 mL of methanol extract was added with 1.25 mL of distilled water and 75 μL of 5% sodium nitrate solution and dissolved for 6 min. Then, 0.5 mL of 1 M NaOH and distilled water were added to the solution until it reached a volume of 2.5 mL. The extract was measured the absorbance values using a UV spectrophotometer at a wavelength of 510 nm (BOECO S-22, Germany). Flavonoid content was obtained by calculating absorbance values using a linear regression equation based on the standard of quercetin and kaempferol.

RESULTS AND DISCUSSION

Biomass of *G. procumbens* callus cultured for 28 days can be seen in Figure 1. The best callus growth was obtained in the combination treatment of 3% and 5% sucrose and 15 min immersion at 12 h interval. Both treatments had almost the same fresh and dry weights and the final biomass obtained after 28 days cultivation increased 2.3 times of the initial inoculum. However, the combination treatment of 3%, 5%, and 7% sucrose and 5 min immersion at 3 h interval did not experience much increase in biomass, even in the addition of sucrose 3%, callus biomass only increased to 0.58 g.

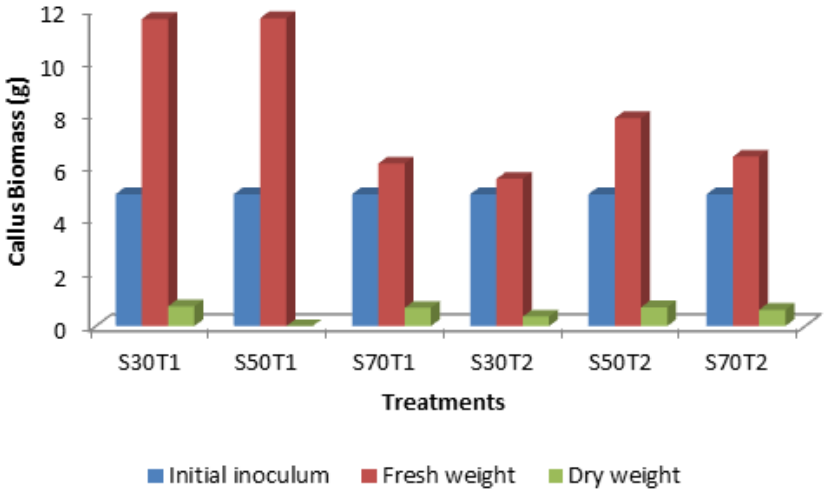

Fig-1: Callus biomass of *G. procumbens* callus in the treatments combination of various sucrose concentration (S30: sucrose 3%, S50: sucrose 5%, S70: sucrose 7%) and immersion frequency (T1: 15 min immersion at 12 h interval; T2: 5 min immersion at 3 h interval) after 28 days cultured

At the end of the culture, callus growth was also followed by the growth of roots and roots formed were green, especially in the treatment of 15 min immersion at 12 h interval (Figure 2). It was happened because the callus was exposed by light continuously causing chlorophyll to form, but it was not happened in the treatment of 5 min immersion at 12 h interval (Figure 3).

The results of this study showed that the combination treatment of 5% sucrose with immersion frequency of 15 min at 12 h interval (S50T1) produced the highest callus biomass. Sucrose at 5% was the optimum concentration to support callus growth. It was indicated by the increase in callus biomass at the end of the treatment that was 2.3 times of the initial inoculums. In the treatment, cells were able to absorb macro and micronutrients that existed in the culture medium, so that callus growth became faster than the other treatments. Besides it, the addition of 5% sucrose into the medium did not affect the osmotic pressure of the cell, so that sucrose could also be maximally absorbed by the cell. The increasing concentration of sucrose will increase the rate of cell respiration in the process of glycolysis. This respiration functions in the anabolic processes and compound synthesis for cell growth [26].

The increase in callus biomass which had similar result was also found in the combination treatment of 3% sucrose with immersion frequency of 15 min at 12 h interval (S30T1). The immersion frequency of 15 min at 12 h interval was able to increase callus biomass compared to the immersion frequency of 5 min at 3 h interval. It is might be caused, the period of immersion too often (8 times per day) gives less chance for callus to do respiration because when the callus is submerged in the medium, the cells do not get oxygen, so that the cells became asphyxia, consequently the cell metabolism is disrupted, on the contrary, a rare immersion period (3 times per day) will give the cell a chance to get the oxygen, so that the cell can do respiration properly and do not became asphyxia. In *G. procumbens* adventitious root culture and *Talinum paniculatum* adventitious root culture, the combination treatment of 5% sucrose and immersion frequency of 15 min at 12 h interval also produced the highest biomass [21, 27].

The addition of 7% sucrose combined with immersion frequency of 15 min at 12 h interval and immersion frequency of 5 minutes at 3 hours interval produced low callus biomass. It is might be caused an increase concentration of sucrose in the plant cell environment will cause the cell lose a lot of water from the cell, because osmotic pressure inside the cell is lower than the osmotic pressure outside the cell so that the cell turgor decreases, this condition will trigger cells to experience plasmolysis [28] and interfere with cell enzymatic processes [16]. The similar result was also found in adventitious root cultureof *G. procumbens* and in adventitious root culture of *Morinda citrifolia* where the decreased biomass was found in the treatment of 7% and 9% sucrose [26, 29].

Morphological observation of callus growth for 28 days in temporary immersion bioreactor showed the occurrence of callus differentiation to form adventitious roots. The emerging of the adventitious root is the response to the use of plant growth regulator (IBA). Adventitious roots formed are green because of the exposure to the light during cultivation. The change of color in the adventitious root is a result of proplastids contained in the callus meristem cells will turn into chloroplasts that contain chlorophyll.

Fig-2 *G. procumbens* callus cultured in temporary immersion bioreactor after 28 days under treatments of various sucrose concentration (3%, 5%, 7%) and immersion frequency of 15 min at 12 h interval.

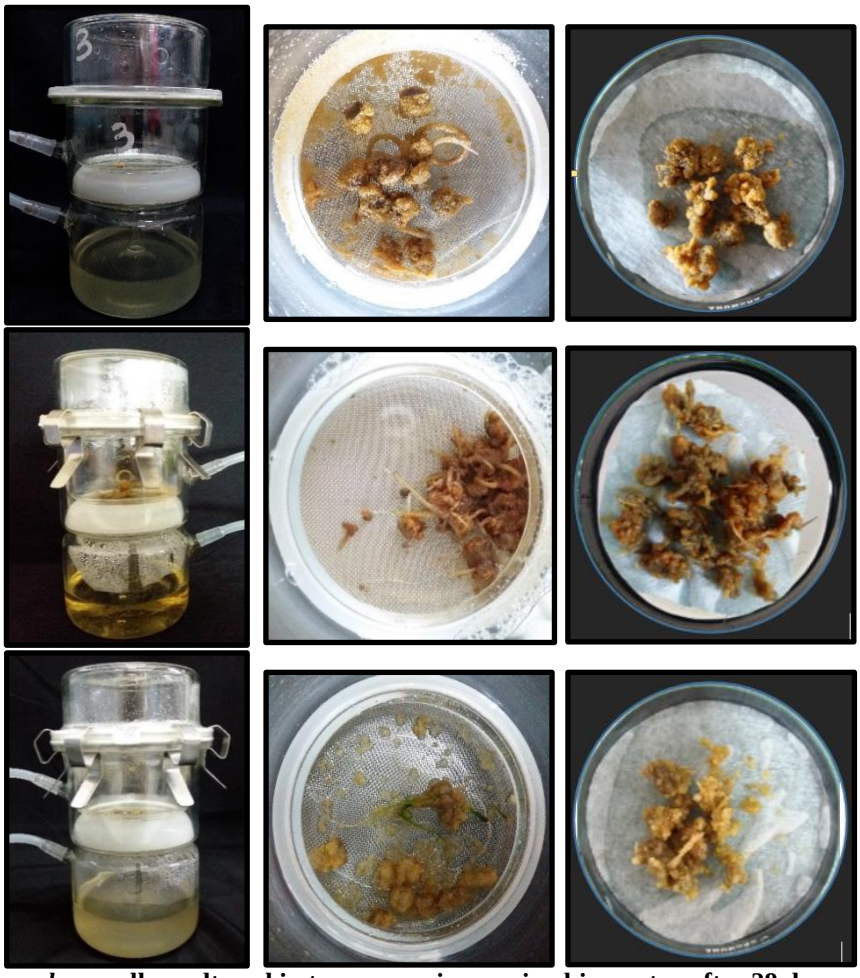

Fig-2: *G. procumbens* callus cultured in temporary immersion bioreactor after 28 days under treatments of various sucrose concentration (3%, 5%, 7%) and immersion frequency of 5 min at 3 h interval

The highest total flavonoid content (kaempferol and quercetin) was obtained in the combination treatment of 7% sucrose and 15 min immersion at 12 h interval, while the lowest total flavonoid content was found in the combination treatment of 3% sucrose and 5 min immersion at 3 h interval (Figure 4). In the combination treatment of 3%, 5%, and 7% sucrose and 5 min immersion at 3 h interval, the increasing concentration of sucrose increased the content of kaempferol and quercetin.

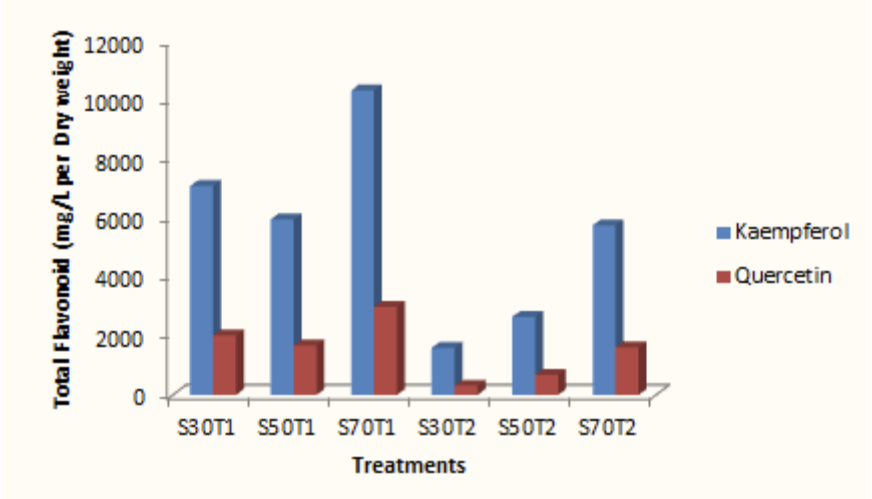

Fig-4: Total flavonoid of *G. procumbens* callus in the treatments combination of various sucrose concentration (S30: sucrose 3%, S50: sucrose 5%, S70: sucrose 7%) and T1 (15 min immersion at 12 h interval); T2 (5 min immersion at 3 h interval) after 28 days cultured

The results of total flavonoid analysis (kaempferol and quercetin) showed that the highest total flavonoid was obtained in the combination of 7% sucrose and immersion frequency of 15 minutes at 8 hours interval. Excess or lack of sucrose in plant cells can cause osmotic stress thereby triggering the formation of secondary metabolites [30].The addition of high sucrose causes the cell to experience osmotic stress so that the production of secondary metabolites is higher. The addition of 4% sucrose was also able toincrease thesecondary metabolite production in *Picrorhiza kurroa* hairy root culture [31].

The use of sucrose may affect the flavonoid biosynthesis pathway on the phenylpropanoid pathway. Sucrose is an essential substance as a donor of carbon skeleton for phenylpropanoid metabolism and as a signaling molecule to increase phenylpropanoid biosynthesis [32]. Beside sucrose, oxygen supply that occurs due to immersion frequency of 15 min at 12 h interval may lead to the effectiveness of time setting to provide nutrients, carbon and oxygen sources. The presence of nutrients, sucrose, and oxygen in the medium and culture environment will be utilized by callus for growth through primary metabolism. Primary metabolism is an activity to fulfill the basic functions of cell life, including cell division, respiration, storage, and reproduction. Besides that, primary metabolites will produce intermediate compounds which are precursors of secondary metabolites.

The results of the total flavonoid analysis of *G. procumbens* callus cultured for 28 days also showed that of all treatments, the content of kaempferol was higher than the content of quercetin. The higher kaempferol formed because the flavonoid biosynthesis pathway started from the shikimate pathway with phenylalanine precursor forms kaempferol earlier than quercetin [33].

CONCLUSION

The production of *G. procumbens* callus biomass cultured in a temporary immersion bioreactor increased 2.3 times of the initial inoculum. The highest increase in biomass was obtained in the combination treatment of 5% sucrose and immersion frequency of 15 min at 12 h interval. The highest total flavonoid (kaempferol and quercetine) production was obtained in a combination of 7% sucrose and immersion frequency of 15 min at 12 h interval.

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