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Optimization of Biomass and Secondary Metabolite Production in *Gynura procumbens* (Lour.) Merr. Adventitious Roots Culture by Using the Method of Subculture and Fed-batch Cultivation in a Bioreactor

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ABSTRACT

The valuable extract of bioactive compounds from *Gynura procumbens* has been widely manufactured into various health products. The demand for these compounds is continuously increasing, but production through conventional farming methods is insufficient due to limited agricultural land and environmental stresses. An alternative to producing plant biomass is in vitro cultivation methods. This method requires less space and enables biomass propagation in a controlled condition that can facilitate stable and efficient production of plant secondary metabolites. This study evaluated the effect of inoculum subculture periods and culture methods on *G. procumbens* biomass and secondary metabolite production in a bioreactor. The 3-L airlift balloon type-bubble bioreactors was modified in this study to adopt the treatment of 1st-5th subculture periods and fed- and batch-cultivation strategies. We found the *G. procumbens* adventitious root culture was optimally derived from the 1st subculture produced biomass of 148.02±1.45 g FW and 8.59±0.12 g DW, and TPC (14.48±1.08 mg GAE/g DW) and TFC (116.89±0.44 mg KE/g DW and 33.97±0.13 mg QE/g DW). Additionally, the fed method after 28 days of culture using double distilled water replenishment improved adventitious root biomass (213.75±35.00 g FW and 11.21±0.18 g DW), while nutrient replenishment improved TFC (52.14±0.44 mg KE/g DW and 14.54±0.13 mg QE/g DW). These results can be used to optimize the cultivation of *G. procumbens* adventitious roots in a large-scale bioreactor.

1. Introduction

Gynura procumbens Lour. (Merr.) is a native Indonesian plant commonly referred to as sambung nyawa (Hidayat 1997). In South-East Asian countries such as Indonesia, Malaysia, and Thailand, the plant is traditionally devoured as a vegetable (Kaewseejan *et al.* 2015). *G. procumbens* is also used in herbal medicine to treat a variety of illness (Manogaran *et al.* 2019). Its organs contain a variety of bioactive compounds with known biological activities, primarily the flavonoids (Jiao-jiao *et al.* 2017) and phenolics (Rosidah *et al.* 2008). Meanwhile, bioactive compounds like cerebroside (Hu *et al.* 2019) and higher levels of flavonoids and phenolics (Krishnan *et al.* 2015) were found particularly in the roots. Previous studies found some phenylpropanoids (caffeic acid and chlorogenic acid) (Saiman *et al.* 2012) and flavonoids (myricetin, kaempferol, quercetin, and catechin) (Kusuma *et al.* 2021) in adventitious root *in vitro*.

Previous works have reported on the pharmaceutical activities of *G. procumbens* extract, especially their positive effect on human health (Takanashi *et al.* 2019). Recent studies have shown that *in vitro* roots of *G. procumbens* have excellent antioxidant activity and hepatoprotective effects (Sugiharto *et al.* 2021, 2022). Furthermore, *G. procumbens* is a safe and edible plant because it

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does not contain toxic compounds like, pyrrolizidine alkaloids (Saiman *et al.* 2012). Therefore, *G. procumbens* extract has been manufactured into end products, such as cosmetics (Seong-in 2018).

The bioactive compounds are mainly extracted from plant biomass restricted by limited suitable agricultural land and various environmental stresses. A possible alternative is the production of plant biomass and metabolites through *in vitro* method (Murthy *et al.* 2014). This method can be scaled-up using a bioreactor culture system to increase the production of target secondary metabolites (Baque *et al.* 2012). Since the highest bioactive compound accumulation was detected in the *G. procumbens* roots, it is desirable to use its adventitious roots as an initial culture to produce secondary metabolites. Although aeration rate and inoculum density in a 3-L bioreactor positively increased the biomass and TFC of *G. procumbens* roots (Kusuma *et al.* 2021) thus the effect of subculture treatment and feed-batch culture in *G. procumbens* adventitious roots culture using a bioreactor has yet to be investigated.

Subculture is required to avoid the accumulation of toxic metabolites (such as carbon dioxide, ethylene, and nitric oxide) and the exhaustion of the medium due to the plant propagule growth in a closed vessel (George 2008). Optimizing the subculture period demonstrated obtaining the stability of biomass on *G. officinalis* hairy roots (Khezri *et al.* 2022) and ginsenoside production of *P. notoginseng* (Zhao *et al.* 2020). However, García-Mateos *et al.* (2005) reported that prolonged subculturing could decrease alkaloid content in *E. americana* callus, suggesting that auxin (2,4-dichloro phenoxy acetic acid) in the media might inhibit the accumulation of alkaloids *in vitro*. Sharma *et al.* (2014) mentioned the adventitious root that underwent several subculture processes was generally used as inoculum in the bioreactor culture. However, there is still no information regarding the effect of subculture periods on biomass and bioactive compound production of *G. procumbens* adventitious root culture in a bioreactor.

The batch culture method is frequently used for culturing adventitious roots in bioreactors. In contrast, the fed-batch culture is commonly used to solve nutrient limits during culture and to enhance biomass and secondary metabolite production (Baque *et al.* 2012). Several studies have reported successful biomass and secondary metabolite production improvement through the fed-culture strategy (Jin *et al.* 2020; Wu *et al.* 2007).

Flavonoids, such as kaempferol and quercetin, are the main compounds in *G. procumbens*. Hence, this study analyzed the effect of periodical subcultures and different culture strategies in a bioreactor on *G. procumbens* adventitious roots biomass and flavonoids (kaempferol and quercetin) production. The adventitious roots used as inoculum starters derived from one to five times subcultured. The best result obtained from subculture treatment was then used to evaluate the effect of different culture strategies, especially between batch and fed-batch cultivations with different media replenishment.

2. Materials and Methods

2.1. Plant Material

Gynura procumbens stem (node and internodes) from one mother plant was used as an explants source. Plant material was prepared and sterilized based on Kusuma *et al.* (2021). The explants were cultured on Murashige and Skoog (MS) solid media containing 5 mg/L indole-3-butyric acids (IBA) (Merck, Germany), 30 g/L sugar, 6 g/L agar, and pH was adjusted to 5.8. The media were sterilized at 121°C for 15 min. The explants were incubated at 25°C and placed in dark conditions for 3 weeks. The roots were harvested and cut into 1–2 cm lengths for the subsequent subcultures.

2.2. Cultivation of Adventitious Root Lines from Periodic Subculture in the Bioreactor

The 3-week-old regenerated adventitious roots were cut into pieces. About 0.2 g of fresh weight (FW) root pieces were transferred into a flask containing 100 ml liquid medium with the same composition for 4 weeks (the 1st subculture). The flasks were shaken with an orbital shaker (IKA-KS130) at 100 rpm and incubated at 25°C in the dark. The regenerated roots from the 1st subculture were used as starter inoculum for the bioreactor. The roots were also transferred into another flask for the subsequent (2nd) subculture. The process was repeated until the 5th subculture. The bioreactor cultivation was performed according to Kusuma *et al.* (2021). The overall method is described in Figure 1.

2.3. Cultivation of Adventitious Root in the Fed-Batch Bioreactor

The 4-week-old adventitious roots from suspension culture were cut into pieces. The 3-L capacity airlift balloon type-bubble bioreactors

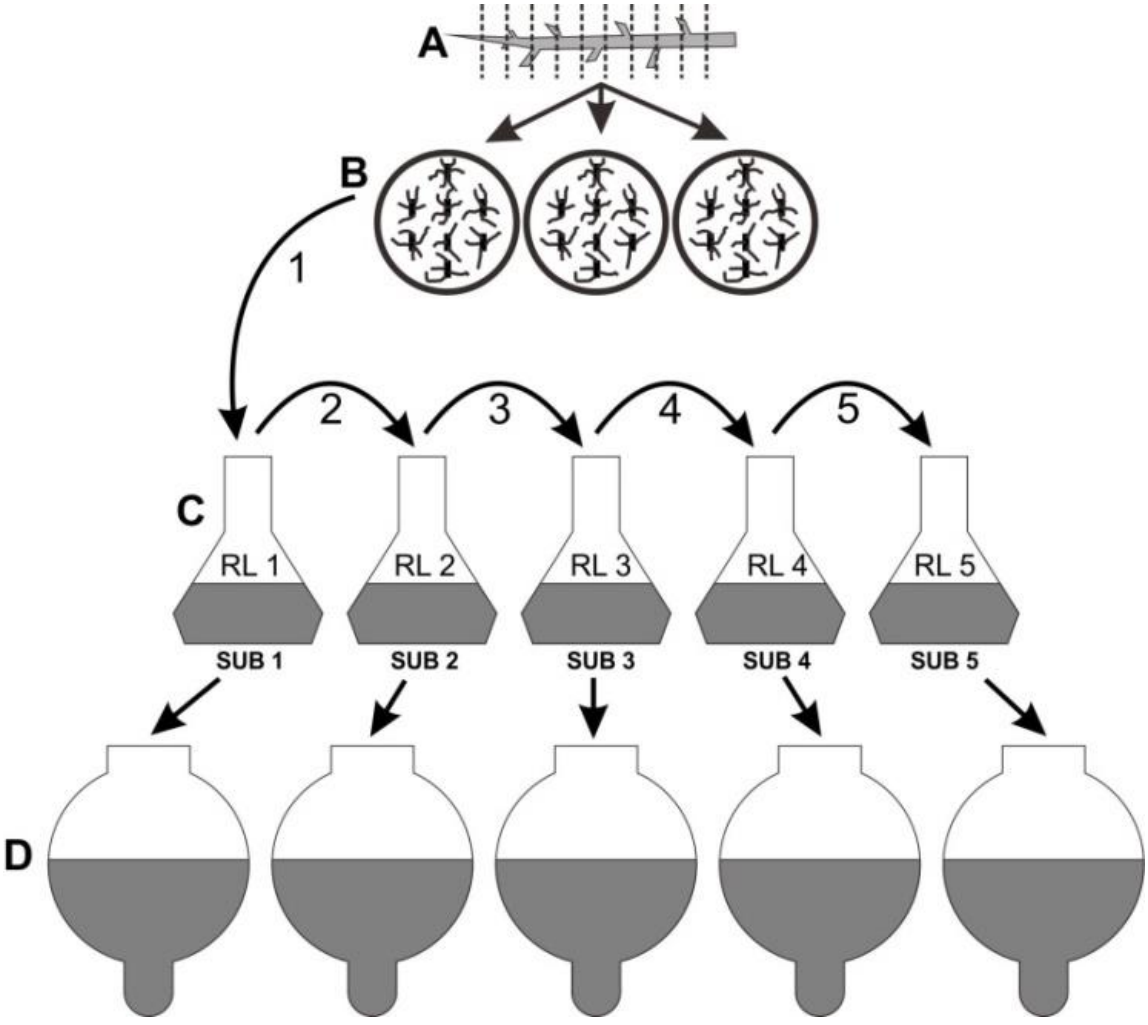

Figure 1. Schematic diagram of various root line cultivation methods from periodic subcultures into bioreactors. (A) Explant sources (parts of the stem), (B) initiation stage, (C) proliferation stage (roots suspension culture in flasks), and (D) bioreactor culture. Numbers 1-5 represent the subculture period. The RL (root line) 1-5 represents root stock from different subculture periods (SUB 1-5). Arrows represent the flow of culture

(BTBB) were modified in this study to adopt fed cultivation and batch cultivation strategies. The schematic set of adopted bioreactors is described in Figure 2. A total of 3 g/L initial inoculums and aeration 0.15 v/vm were performed according to Kusuma *et al.* (2021) for control treatment and fed cultivation in a bioreactor. For the fed-cultivation system, 500 ml of liquid MS media and double distillate water (DDW) were added into the bioreactor on the 28th day after culture, respectively. Optimizing the culture period for batch and fed cultivations were performed for 56 days and maintained at 25 °C in dark conditions.

2.4. Biomass Measurement

The roots were washed with tap water and drained on filter paper (3 times). The FW of adventitious roots was determined by weighing fresh roots with an analytical balance (Shimadzu-LIBROR AEL200). Further, the roots were air-dried at room temperature (30-34°C) to determine the dry weight (DW).

The dry roots were ground into a powder using a pestle and mortar. The DW was determined by weighing root powder using an analytical balance. The growth ratio was calculated using the following formula:

Growth ratio = (Harvested roots (DW)) – (Root initial inoculum (DW)) / (Root initial inoculum (DW))

2.5. Phenolic and Flavonoid Extraction

According to Kusuma *et al.* (2021), about 1 g of root powder from each treatment was macerated with 5 ml (1:10 w/v) 70% methanol (Merck, Germany). The

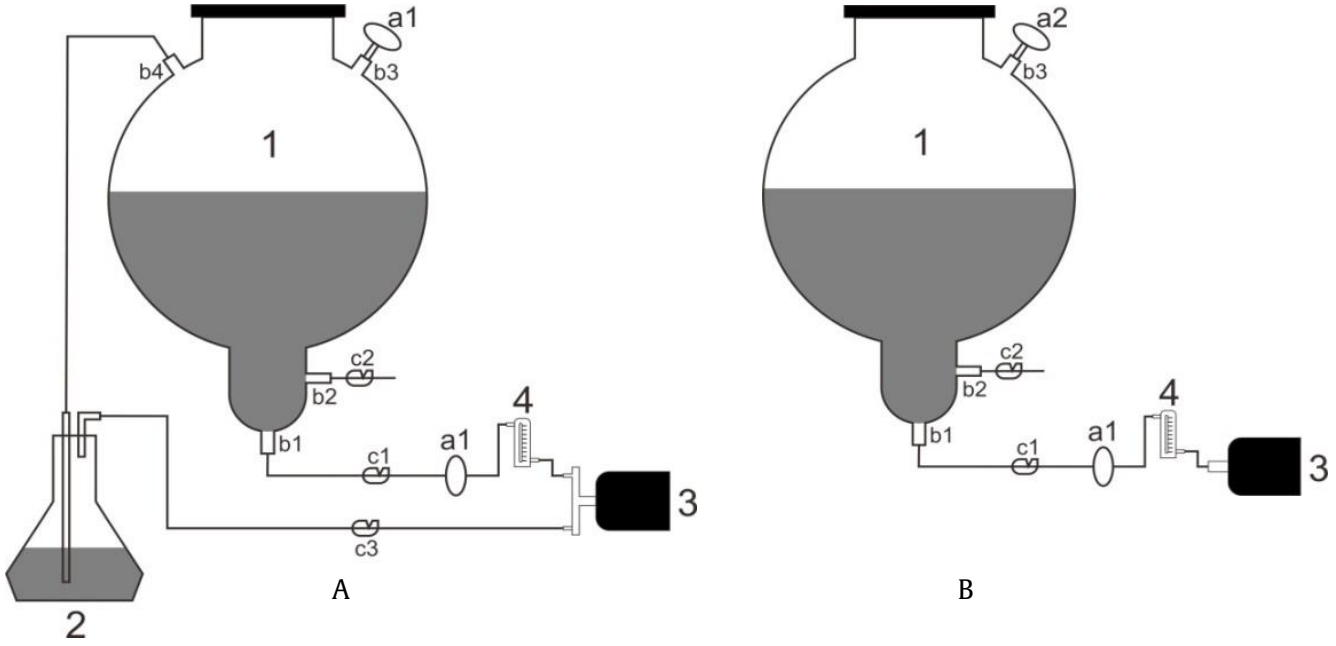

Figure 2. Schematic diagram of bioreactor installation for (A) fed cultivation and (B) batch cultivation. (1) bioreactor vessel, (2) flask of the medium reservoir, (3) air pump, and (4) airflow meter. The filter membrane was 0.2 µm (a1 and a2). The bioreactor ports: (b1) air inlet port, (b2) port of medium sampling, (b3) air outlet port, and (b4) nutrient-fed inlet port. The hose clamps open or close the airflow (c1 and c3) and the medium sample (c2). The nutrient-fed processes were: the inlet airflow towards the bioreactor was closed by a clamp (c1) and opened airflow clamp (c3) towards the flask. The liquid medium will flow into the bioreactor through the nutrient-fed port inlet (b4).

macerated solutions were shaken with an orbital shaker (DAIHAN LABTECH-LSI3016A) at 90 rpm and incubated at 28°C. This procedure was repeated twice. Filter paper was used to separate the crude liquid extracts from the solid debris. As much as 10 mL of crude liquid extracts from each treatment sample were used for total phenolic (TPC) and total flavonoid (TFC) measurements.

2.6. TPC and TFC Measurement

TPC was analyzed based on the methods by Kaewseejan *et al.* (2015). As much as 200 µL of sample extract was added to 1 ml of Folin–Ciocalteau reagent (Merck, Germany) (1:10 with dH_2O) and left for 5 min. Then 0.8 mL of 7.5% Na_2CO_3 (Merck, Germany) solution was added and allowed to stand for 30 min at room temperature. The blank solution was 70% methanol, treated the same as the test sample. The measurement of TPC used a UV-vis spectrophotometer (BOECO S-22, Germany) at 765 nm. The concentration of TPC is equivalent to a milligram of gallic acid standard (mg GEA) (Merck, Germany). The measurement results were converted and expressed in units of mg GEA/g DW.

TFC was analyzed based on the methods by Kaewseejan *et al.* (2015). 250 µL sample extract mixed with 1,250 µL dH_2O and 75 µL of 5% (w/v) $NaNO_2$ (Merck, Germany). The mixture was incubated for 5 min and then adding 150 µL of 1% (w/v) AlCl3 (Merck, Germany). Next, 150 µL of 1 M NaOH (Merck, Germany) was added and dH_2O to a final volume of 2,500 µL. The blank solution was 70% methanol, treated the same as the test sample. TFC was measured using a UV-vis spectrophotometer at 510 nm. The concentration of TFC is equivalent to a milligram of quercetin (mg QE) and kaempferol (mg KE) (Sigma-Aldrich, USA). The measurement was converted and expressed in mg QE/g DW and mg KE/g DW, respectively.

2.7. Measurement of Malondialdehyde (MDA) Contents

MDA measurement was performed using a modified method based on Zhang and Huang (2013). As much as 0.5 g of fresh roots sample was homogenized using a mortar with 4.5 ml of 1% H_2SO_4 (Merck, Germany). The homogenate was centrifuged at 6,000 rpm for 10 minutes. Then 1 ml of the

supernatant was mixed with 0.5 ml of 0.1% TCA (Merck, Germany), and 2 ml of 20% TCA + 0.5% TBA (Merck, Germany). The solution was heated at 100°C for 60 minutes and cooled quickly for 30 minutes. The blank solution was given the same treatment without the root sample. The absorbance was measured at 532 nm using a UV-vis spectrophotometer. The MDA concentration was determined using a standard curve created with MDA standard solution (0–40 nmol). The results were given in units of nmol/0.5 g FW.

2.8. Measurement of Proline Contents

Proline was measured based on Bates *et al.* (1973). About 0.5 g of fresh roots sample was homogenized using a mortar with 4,500 µL of 3% sulfosalicylic acid solution (Merck, Germany). The homogenate was centrifuged at 6,000 rpm. Next, 1,000 µL of ninhydrin acid solution (Merck, Germany) was reacted with 500 µL of the sample supernatant. The solution was heated for 60 minutes at 100°C and then allowed to cool for 30 minutes. The sample solution was added 1,500 µL of toluene, then vortexed for 1 min. A UV-vis spectrophotometer set to 520 nm was used to measure the chromophore. The blank solution used pure toluene. The proline concentration was calculated using an L-proline standard solution (0–300 µM) (Merck, Germany). The results were given in the form M /0.5 g FW.

2.9. Statistical Analysis

A one-way analysis of variance (p0.05) was used to analyze the data, followed by Duncan's multiple range test (DMRT) at a 95% confidence level. The statistical analyses were performed using IBM-SPSS software v.21.

3. Results

3.1. Effects of Inoculum Starters on Root Biomass and Secondary Metabolite Production

Table 1 shows the highest adventitious root FW was achieved from the culture with inoculum from the 5th subculture (155.25±2.12 g), followed by the 2nd (154.93±1.52 g), the 1st subculture (148.02±1.45 g), and the 4th subculture (127.88±2.65 g) (Table 1). The lowest was 3rd subculture (119.75±2.12 g). In contrast, the highest DW was achieved from the culture with inoculum starter from the 2nd subculture (8.64±0.15 g) and a slight decrease in culture with the inoculum starter from the 1st subculture (8.59±0.12 g) (Table 1).

The DW yield decreased in the 4th subculture (7.48±0.12 g) and further decreased in the 3rd subculture (7.41±0.13 g). The lowest DW was recorded in the 5th subculture (7.15±0.10 g). The difference in growth speed was shown by calculating the ratio of adventitious root growth obtained from each treatment (Table 1). We also observed a slight difference in root morphology among the cultures that use inoculum from different subculture periods (Figure 3).

Table 1 shows that the TPC and TFC of each treatment were significantly different. TPC and TFC in the roots harvested from the 1st subculture treatment (14.48±1.08 mg GAE/g DW, 116.89±0.44 mg KE/g DW, and 33.97±0.13 mg QE/g DW, respectively) were significantly higher than other treatments, while the lowest was in the 5th subculture treatment (2.93±0.13 mg GAE/g DW, 27.85±0.34 mg KE/g DW, and 7.26±0.10 mg QE/g DW, respectively). TPC and TFC showed random decline patterns from the 1st to the 5th treatment. However,

Table 1. The biomass production of *G. procumbens* adventitious root was harvested from a culture in a 3-L bioreactor using different inoculum starters from various subculture periods

Cultivation types	Biomass			TPC	TFC	
	FW (g)	DW (g)	GR	(mg GAE/g DW)	(mg KE/g DW)	(mg QE/g DW)
SUB-1	148.02±1.45	8.59±0.12	41.95	14.48±1.08^{e}	116.89±0.44^{e}	33.97±0.13^{e}
SUB-2	154.93±1.52	8.64±0.15	42.20	4.79±0.26^{b}	58.37±1.78^{b}	16.41±0.53^{b}
SUB-3	119.75±2.12	7.41±0.13	36.06	9.07±0.33^{c}	89.26±1.36^{c}	25.68±0.41^{c}
SUB-4	127.88±2.65	7.48±0.12	36.40	11.55±0.40^{d}	95.11±0.44^{d}	27.43±0.13^{d}
SUB-5	155.25±2.12	7.15±0.10	34.76	2.93±0.13^{a}	27.85±0.34^{a}	7.26±0.10^{a}

FW: Fresh Weight, DW: Dry Weight, GR: Growth Ratio
TPC: Total Phenolic content, TFC: Total Flavonoid content
^{a,b,c,d} The mean followed by the different letters is significantly different based on DMRT ($p<0.05$) (n = 3)

the overall results showed decreased secondary metabolite production might be due to increased subculture periods.

3.2. Effects of Cultivation Types on Root Biomass and Secondary Metabolite Production

Based on Table 2, the DDW-fed treatment produced the highest FW (213.75±35.00 g), followed by batch cultivation (174.00±61.52 g), nutrient-fed (169.85±18.60 g), and control treatment (150.80±1.90 g). The highest DW was also achieved from the DDW-fed treatment (11.21±0.18 g), while the lowest was achieved in the control treatment (8.02±0.42 g). The morphology of adventitious roots obtained from different methods is presented in Figure 4.

Different cultivation types did not significantly affect the TFC but significantly affected the TPC (one-way ANOVA test, $p<0.05$). The highest TFC was obtained from the nutrient-fed treatment (52.14±0.44 mg KE/g DW and 14.54±0.13 mg QE/g DW), while the lowest was obtained from the batch cultivation treatment (48.37±1.35 mg KE/g DW and 13.41±0.40 mg QE/g DW). For the TPC, control showed the highest (6.97±0.15 mg GAE/g DW), followed by the batch cultivation (5.18±0.32 mg GAE/g DW), the nutrient-fed (4.72±1.08 mg GAE/g

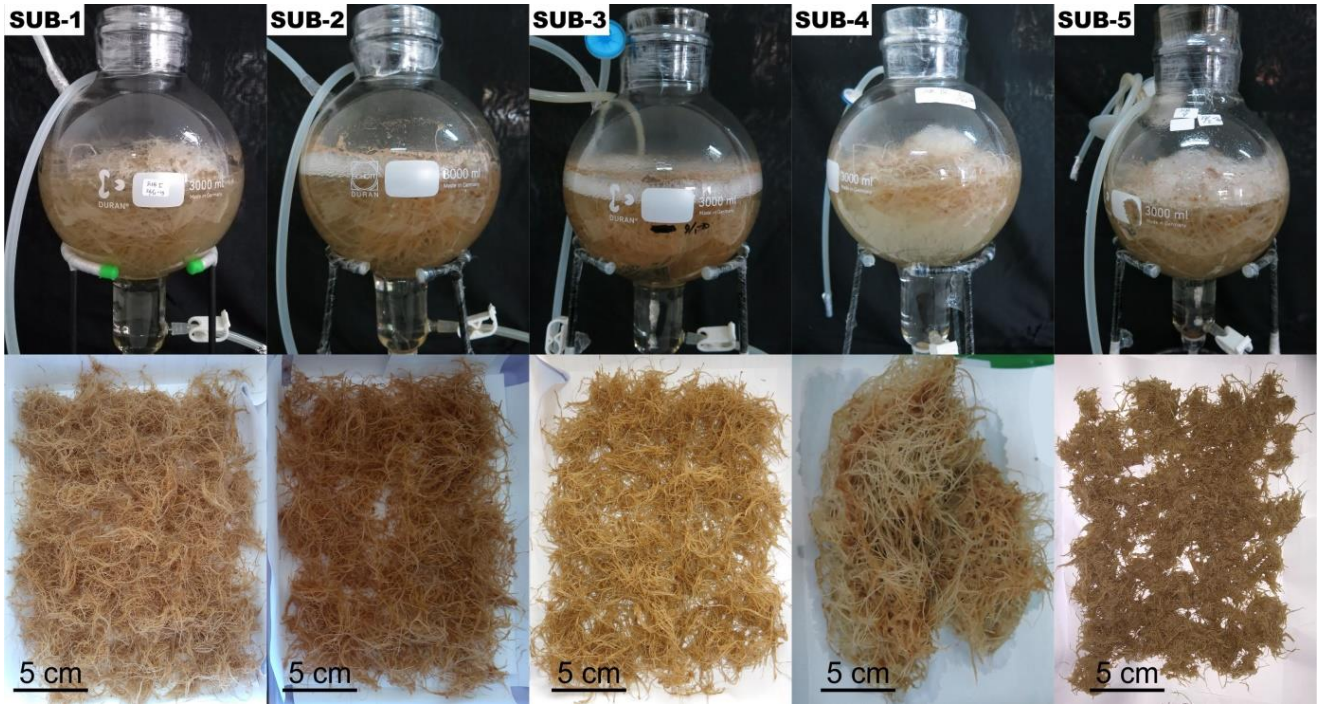

Figure 3. The yield and morphology of *G. procumbens* adventitious root were harvested from a culture in a 3-L bioreactor using different inoculum starters from various subculture periods

Table 2. The biomass production of *G. procumbens* adventitious root was harvested from a culture in a 3-L bioreactor using different cultivation types

Cultivation types	Biomass			TPC	TFC	
	FW (g)	DW (g)	GR	(mg GAE/g DW)	(mg KE/g DW)	(mg QE/g DW)
DDW-fed	213.75±35.00	11.21±0.18	55.05	4.16±0.26[a]	50.59±1.78	14.07±0.53
Nutrient-fed	169.85±18.60	8.92±0.50	43.57	4.72±1.08[b]	52.14±0.44	14.54±0.13
Batch	174.00±61.52	8.17±1.38	39.83	5.18±0.32[b]	48.37±1.35	13.41±0.40
control	150.75±1.90	8.02±0.42	39.10	6.97±0.15[c]	50.00±0.58	13.90±0.17

DDW-fed: Double distillate water fed
FW: Fresh Weight, DW: Dry Weight, GR: Growth Ratio
TPC: Total Phenolic content, TFC: Total Flavonoid content
[a,b,c] The mean followed by the different letters are significantly different based on DMRT ($p<0.05$) ($n = 3$)

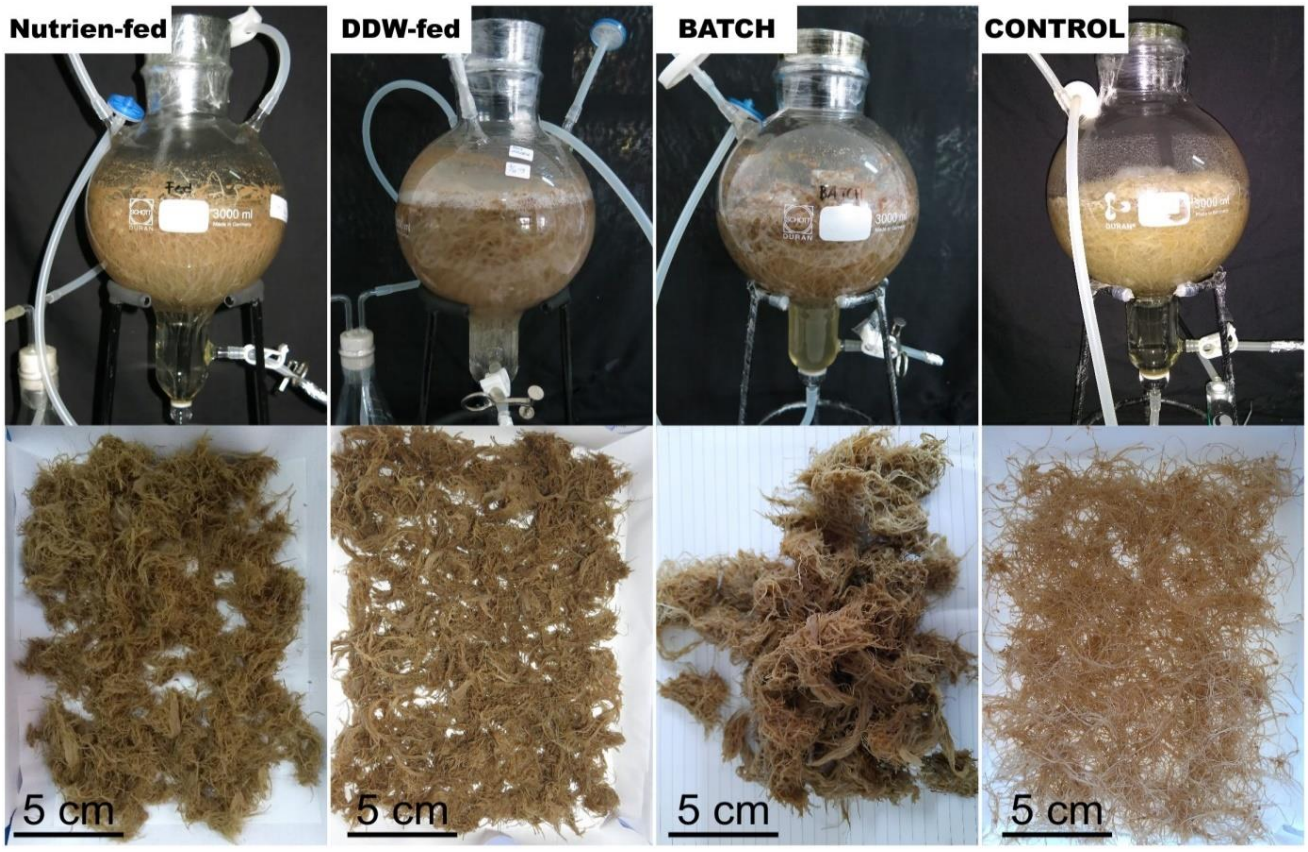

Figure 4. The yield and morphology of *G. procumbens* adventitious root were harvested from a culture in a 3-L bioreactor using different cultivation types

DW), and the DDW-fed treatment (4.16±0.26 mg GAE/g DW) (Table 2).

3.3. Malondialdehyde (MDA) and Proline Levels of Various Inoculum Starters and Cultivation-Type Treatments

MDA was detected in all subculture treatments, with the 1st subculture (5.10±0.12 nmol/0.5 g FW) and the 2nd subculture (5.07±0.08 nmol/0.5 g FW) being significantly higher than other treatments (Table 3). Although the lowest MDA level was recorded from the 5th subculture (4.51±0.08 nmol/0.5 g FW), differences between the 3rd (4.67±0.16 nmol/0.5 g FW), 4th (4.64±0.16 nmol/0.5 g FW) and 5th subcultures were not significant (Table 3).

On the contrary, the proline levels of the adventitious root culture derived from the 5th subculture treatment (209.65±10.63 µM/0.5 g FW) were significantly higher than other treatments (Table 3). The lowest level of proline (123.23±3.72 µM/0.5 g FW) was obtained in the adventitious roots with an inoculum starter from the 1st subculture. In contrast, the proline levels in roots from the 2nd (146.97±8.98 µM/0.5 g FW), the 3rd (158.61±11.87 µM/0.5 g FW), and the 4th (155.19±8.14 µM/0.5 g FW) subculture treatments were not significantly different (Table 3).

The cultivation types significantly affected the MDA and proline levels in the adventitious roots of *G. procumbens* (Table 4). The DDW replenishment treatment produced the lowest level of MDA (4.29±0.09 nmol/0.5 g FW), whereas batch cultivation treatment produced the highest MDA (7.57±0.08 nmol/0.5 g FW). Interestingly, the control produced a higher MDA (5.64±0.08 nmol/0.5 g FW) than the nutrient-fed treatment (5.21±0.16 nmol/0.5 g FW) (Table 4).

The control produced the highest proline level (255.73±11.56 µM/0.5 g FW), whereas the lowest was detected in the DDW-fed treatment (6.41± 6.56 µM/0.5 g FW) (Table 4). The proline level in the nutrient-fed and batch cultivation treatments was 44.73±6.05 and 156.44±6.58 µM/0.5 g FW, respectively.

Table 3. Effect of different inoculum starters from various subculture periods on MDA and proline levels *G. procumbens* adventitious root culture in a 3-L.

Subculture periods	MDA (nmol/0.5 g FW)	Proline (µM/0.5 g FW)
SUB-1	5.10±0.12[b]	123.23±3.72[a]
SUB-2	5.07±0.08[b]	146.97±8.98[b]
SUB-3	4.67±0.16[a]	158.61±11.87[b]
SUB-4	4.64±0.16[a]	155.19±8.14[b]
SUB-5	4.51±0.08[a]	209.65±10.63[c]

[a,b,c] The mean followed by the different letters is significantly different based on DMRT ($p<0.05$) ($n = 3$)

Table 4. Effect of different cultivation types on MDA and proline levels *G. procumbens* adventitious root culture in a 3-L

Cultivation type	MDA (nmol/0.5 g FW)	Proline (µM/0.5 g FW)
DDW-fed	4.29±0.09[a*]	6.41±6.56[a]
Nutrient-fed	5.21±0.16[b]	44.73±6.05[b]
BATCH	7.57±0.08[d]	156.44±6.58[c]
Control	5.64±0.08[c]	255.73±11.56[d]

DDW-fed: Double distillate water fed

[a,b,c,d] The mean followed by the different letters is significantly different based on DMRT ($p<0.05$) ($n = 3$)

4. Discussion

4.1. The Effect of Various Inoculum Starter Treatments on Biomass and Secondary Metabolite Production

In this study, the derived adventitious root lines from periodical subcultures generated different yields of root biomass, indicating that the periodical subcultures could influence the adventitious root proliferation capability (Table 1). There was a slight difference in root proliferation. The adventitious roots in the 1st to the 3rd subculture have thicker and longer root branches than those from the 4th and the 5th subcultures. Furthermore, in the 4th and 5th subcultures, some calli were formed within root branches inside the root rolls. The thick roots with a brownish-yellow color were more abundant than the bright-yellowish color thin roots (Figure 3).

Therefore, high concentrations of IBA might affect the proliferation of adventitious root lines during periodic subcultures. The formation of calli in the 4th and 5th subculture periods might be caused by an accumulation of the hormone in the explants during continuous subculture in media containing high IBA. In the present study, 5 mg/L of IBA was the optimal concentration to induce *G. procumbens* adventitious roots. Based on the results, the direct adventitious root formation occurred in the 1st to the 3rd subcultures but tended to form calli in the 4th and the 5th subcultures. Besides inducing adventitious root formation, adding IBA into a medium can induce callus formation and differentiate into the adventitious roots (Ludwig-Müller *et al.* 2005). For instance, 5 mg/L of IBA could form high calli around root branches of the *G. procumbens* (Kusuma *et al.* 2017) and *M. citrifolia* (Baque *et al.* 2010) adventitious root culture. The calli formation on roots might increase water uptake, as shown in the FW of the roots from the 5th subculture treatment (Table 1).

Related to the above, the subculture cycle of the inoculum is one of the key factors affecting the production of biomass and bioactive compound in a bioreactor (Kim *et al.* 2002). This study's different subculture periods influenced the secondary metabolite levels in adventitious roots. We assume that saturation of IBA during subculture periods might develop a specific response to the IBA, affecting the accumulation of secondary metabolites. For example, an IBA at high concentrations (5 mg/L) could reduce TPC and TFC (Jang *et al.* 2012). Auxin saturation might inhibit flavonoid biosynthesis and accumulation in a specific location (Besseau *et al.* 2007; Morales-Quintana and Ramos 2021). However, it is unclear if the exogenous IBA influenced the accumulation of secondary metabolites during periodical subcultures.

On the other hand, the stress condition in liquid media might affect flavonoid accumulation and transport of auxin (Ferreyra *et al.* 2012). As shown in Table 3, high levels of MDA were recorded in each subculture, suggesting that the submerged roots in liquid media encountered oxidative stress due to aeration shear stress. Root cells then activate their defense mechanisms by increasing antioxidant activities (Baque *et al.* 2013) and producing phenolic and flavonoid compounds (Ferreyra *et al.* 2012).

4.2. The Effect Of Cultivation-Type Treatments On Biomass And Secondary Metabolite Production

This is the first report for *G. procumbens* adventitious root culture using the fed-batch method. The results follow previous studies on other cultures, such as *P. ginseng* (Jeong *et al.* 2008), and *O. elatus* (Jin *et al.* 2020). We found that replenishment using DDW produced higher biomass than other treatments.

The DDW-fed might help dilute the media because media become concentrated after some time due to aeration and water absorption. The concentrated media might affect root growth and nutrient uptake.

On the other hand, the availability of nutrients and sugar is critical for long-term culture. Jeong *et al.* (2008) reported that NH_4^+ and HPO_4^- were wholly depleted in the culture media within 20-30 days, whereas macronutrients (K^+, Cl^-, SO_4^-) and sugar were drastically reduced. Without medium replenishment, the authors found that the root growth slowed after 4 weeks of culture. Therefore, replenishing media is desirable for the growth of long-term adventitious root cultures.

An increase in the yield of bioactive compounds using the fed-culture method was observed on the adventitious root culture of *G. procumbens*, probably due to media replenishment. TFC from the fed culture (nutrient and DDW) was higher than the culture without medium replenishment (batch cultivation). However, the TPC in the batch culture was higher than in both medium replenishment treatments (Table 2). A similar finding has been reported by Wu *et al.* (2007), where the TPC and TFC on cultures with replenishment media were lower than the control (without medium replenishment). However, the authors indicated that replenishment media could increase the production of caffeic acid derivatives. In this study, the control was superior to others in producing TPC, while the nutrient-fed treatment was superior in the TFC (although not significantly different).

In this study, the 56-day root cultures did not produce higher secondary metabolites than the 28-day cultures. Therefore, further investigation is required to determine this. In addition, modifying the fed-batch culture method by replenishing the medium at the end of 2 weeks after the inoculation process (Wu *et al.* 2007) or reducing the medium volume at the initial culture (Jin *et al.* 2020) might be of interest as these parameters have been shown to improve the accumulation of secondary metabolites in other cultures.

4.3. The Effect of Various Inoculum Starters and Cultivation Types on Malondialdehyde (MDA) and Proline Contents

The damage to cells membrane due to lipid peroxidation can be seen through the accumulation of MDA (Baque *et al.* 2014). In this study, high levels of MDA were detected when using inoculum from the 1st and 2nd subcultures, probably due to the low phenol accumulation that could not cope with the oxidative stress. A similar phenomenon has also been reported in *P. multiflorum* (Ho *et al.* 2017).

Different cultural methods affected the MDA levels. Many blackish roots were observed in the root roll on batch cultivation, indicating cell damage during culture. Lee *et al.* (2011) reported a high percentage of death in high-density *E. koreanum* Nakai adventitious root cultures. In addition, Cui *et al.* (2011) reported that increased culture duration increased MDA levels in *H. perforatum* roots.

Aeration might cause increased residual salts in the medium in a bioreactor, leading to free proline accumulation in the root tissues (Sakamoto and Murata 2002). Hence, replenishing MS medium and DDW might dilute the culture media, resulting in low proline content (Table 4). A similar finding has been reported by Baque *et al.* (2014), where the authors found that proline content was influenced by media salt strength in *M. citrifolia* roots culture (Baque *et al.* 2014). However, high proline accumulation might result in a higher TPC.

In conclusion, we found that the adventitious root lines from the first subculture and the DDW-fed treatment 28 days after culture could increase biomass and secondary metabolite accumulations in the *G. procumbens* adventitious roots. This study is a foundation for future studies to develop large-scale cultivation for producing *G. procumbens* adventitious roots and secondary metabolites.

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References

Baque, M.A., Hahn, E.J., Paek, K.Y., 2010. Induction mechanism of adventitious root from leaf explants of *Morinda citrifolia* as affected by auxin and light quality. *In Vitro Cell Dev Biol Plant*. 46, 71-80. https://doi.org/10.1007/s11627-009-9261-3

Baque, M.A., Moh, S.H., Lee, E.J., Zhong, J.J., Paek, K.Y., 2012. Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. *Biotechnol. Adv.*, 30, 1255-1267. https://doi.org/10.1016/j.biotechadv.2011.11.004

Baque, M.A., Murthy, H.N., Paek, K.Y., 2014. Adventitious root culture of *Morinda citrifolia* in bioreactors for production of bioactive compounds, in: Paek, K., Murthy, H., Zhong, J. (Eds.), *Production of biomass and bioactive compounds using bioreactor technology*. Dordrecht: Springer, pp. 185–222. https://doi.org/10.1007/978-94-017-9223-3_9

Baque, M.A., Shiragi, M., Kabir, H., Moh, S.H., Lee, E.J., Paek, K.Y., 2013. Production of biomass and bioactive compounds by adventitious root suspension cultures of *Morinda citrifolia* (L.) in a liquid-phase airlift balloon-type bioreactor. *In Vitro Cell Dev Biol Plant*. 49, 737-749. https://doi.org/10.1007/s11627-013-9555-3

Bates, L., Waldren, R., Teare, I., 1973. Rapid determination of free proline for water-stress studies. *Plant Soil*. 39, 205-207. https://doi.org/10.1007/BF00018060

Besseau, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B., Legrand, M., 2007. Flavonoid accumulation in Arabidopsis repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell*. 19, 148-162. https://doi.org/10.1105/tpc.106.044495

Cui, X.H., Murthy, H.N., Jin, Y.X., Yim, Y.H., Kim, J.Y., Paek, K.Y., 2011. Production of adventitious root biomass and secondary metabolites of *Hypericum perforatum* L. in a balloon type airlift reactor. *Bioresour. Technol.* 102, 10072-10079. https://doi.org/10.1016/j.biortech.2011.08.044

Ferreyra, M.L.F., Rius, S.P., Casati, P., 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Front. Plant Sci.* 3, 222. https://doi.org/10.3389/fpls.2012.00222

García-Mateos, M., Soto-Hernandez, R., Gutierrez, R., Villegas-Monter, A., 2005. Alkaloids from several subcultures of *Erythrina americana* Miller calluses. *Rev Chapingo Ser Hortic.* 11, 21-26. https://doi.org/10.5154/r.rchsh.2003.09.051

George, E.F., 2008. Plant tissue culture procedure-background, in: George, E.F., Hall, M.A., Klerk, G.J.D. (Eds.), *Plant propagation by tissue culture*. Dordrecht: Springer, pp. 502. https://doi.org/10.1007/978-1-4020-5005-3

Hidayat, S., 1997. Study of compositae in several conservation areas in indonesia. *Compos Newsl.* 31, 17-20.

Ho, T.T., Lee, K.J., Lee, J.-D., Bhunhan, S., Paek, K.Y., Park, S.Y., 2017. Adventitious root culture of *Polygonum multiflorum* for phenolic compounds and its pilot-scale production in 500 L-tank. *Plant Cell Tissue Organ Cult*. 130, 167-181. https://doi.org/10.1007/s11240-017-1212-9

Hu, J.W., Wu, J., Li, X.H., Huang, B.H., Yuan, E., Wu, L., Ma, G.Q., 2019. A new cerebroside from the roots of *Gynura procumbens. Chem. Nat. Compd.* 55, 1053-1055. https://doi.org/10.1007/s10600-019-02891-5

Jang, Y.S., Cui, H.Y., Lee, E.J., Kim, H.W., Paek, K.Y., 2012. Auxin affects on production of adventitious roots and secondary metabolites in *Echinacea angustifolia. KOREAN J. CROP Sci*., 20, 479-486. https://doi.org/10.7783/KJMCS.2012.20.6.479

Jeong, C.S., Murthy, H.N., Hahn, E.J., Paek, K.Y., 2008. Improved production of ginsenosides in suspension cultures of ginseng by medium replenishment strategy. *J. Biosci. Bioeng*. 105, 288-291. https://doi.org/10.1263/jbb.105.288

Jiao-Jiao, Y., Zhi-Hong, P., Feng-Mei, H., 2017. Simultaneous determination of nine components in *Gynura procumbens* aqueous extract by HPLC-MS/MS. *Chin. Tradit. Herb. Drugs.* 48, 294-298.

Jin, M., Piao, X., Wu, X., Fan, M., Li, X., Yin, C., Lian, M., 2020. *Oplopanax elatus* adventitious root production through fed-batch culture and their anti-bacterial effects. *Plant Cell Tissue Organ Cult.* 140, 447-457. https://doi.org/10.1007/s11240-019-01740-1

Kaewseejan, N., Sutthikhum, V., Siriamornpun, S., 2015. Potential of *Gynura procumbens* leaves as source of flavonoid-enriched fractions with enhanced antioxidant capacity. *J. Funct. Foods*. 12, 120-128. https://doi.org/10.1016/j.jff.2014.11.001

Khezri, M., Asghari Zakaria, R., Zare, N., Johari-Ahar, M., 2022. Improving galegine production in transformed hairy roots of *Galega officinalis* L. via elicitation. *AMB Express*, 12, 1-14. https://doi.org/10.1186/s13568-022-01409-7

Kim, Y., Wyslouzil, B.E., Weathers, P.J., 2002. Secondary metabolism of hairy root cultures in bioreactors. *In Vitro Cell. Dev. Biol. Plant*. 38, 1-10. https://doi.org/10.1079/IVP2001243

Krishnan, V., Ahmad, S., Mahmood, M., 2015. Antioxidant potential in different parts and callus of *Gynura procumbens* and different parts of *Gynura bicolor. Biomed Res. Int.* 2015, 1-7. https://doi.org/10.1155/2015/147909

Kusuma, D.Y., Kristanti, A.N., Manuhara, Y.S.W., 2017. Effect of sucrose and immersion frequency on production of adventitious roots and secondary metabolites of *Gynura procumbens* (Lour.) Merr in temporary immersion bioreactors. *Asian J. Plant Sci.* 16, 24-36. https://doi.org/10.3923/ajps.2017.24.36

Kusuma, D.Y., Kristanti, A.N., Wibowo, A.T., Chin, T.B., Manuhara, Y.S.W., 2021. Aeration volume and inoculum density using in bioreactor to optimized biomass production and secondary metabolites in *Gynura procumbens* (Lour.) Merr. *Syst. Rev. Pharm*. 12, 102-112.

Lee, E.J., Moh, S.H., Paek, K.Y., 2011. Influence of inoculum density and aeration volume on biomass and bioactive compound production in bulb-type bubble bioreactor cultures of *Eleutherococcus koreanum* Nakai. *Bioresour. Technol.* 102, 7165-7170. https://doi.org/10.1016/j.biortech.2011.04.076

Ludwig-Müller, J., Vertocnik, A., Town, C.D., 2005. Analysis of indole-3-butyric acid-induced adventitious root formation on Arabidopsis stem segments. *J. Exp. Bot.* 56, 2095-2105. https://doi.org/10.1093/jxb/eri208

Manogaran, M., Lim, V., Mohamed, R., 2019. Phytoconstituents of the *Gynura procumbens* ethanol leaf extract and its fractions and their effects on viability of macrophages. *J. Herbmed. Pharmacol.* 8, 224-230. https://doi.org/10.15171/jhp.2019.33

Morales-Quintana, I., Ramos, P., 2021. A talk between flavonoids and hormones to reorient the growth of gymnosperms. *Int. J. Mol. Sci.* 22, 12630. https://doi.org/10.3390/ijms222312630

Murthy, H.N., Wu, C.H., Cui, Y.Y., Paek K.Y., 2014. Production of caffeic acid derivatives from adventitious root cultures of *Echinacea purpurea* (L.) Moench, in: Paek, K., Murthy, H., Zhong, J. (Eds.), *Production of Biomass and Bioactive Compounds Using Bioreactor Technology*. Dordrecht: Springer, pp. 167–184. https://doi.org/10.1007/978-94-017-9223-3_8\

Rosidah, Yam, M., Sadikun, A., Asmawi, M., 2008. Antioxidant potential of *Gynura procumbens. Pharm. Biol.* 46, 616-625. https://doi.org/10.1080/13880200802179642

Saiman, M.Z., Mustafa, N.R., Schulte, A.E., Verpoorte, R., Choi., Y.H., 2012. Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *Gynura procumbens. Plant Cell Tissue Organ Cult.* 109, 465-475. https://doi.org/10.1007/s11240-011-0111-8

Sakamoto, A., Murata, N., 2002. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ.* 25, 163-171. https://doi.org/10.1046/j.0016-8025.2001.00790.x

Seong-Jin, P., 2018. Manufacturing method for cosmetics of *Gynura procumbens*. South Korea patent application No. 10-2119326.

Sharma, P., Sharma, S., Yadav, S., Srivastava, A., Purohit, I., Shrivastava, N., 2014. Plant derived bioactive molecules: culture vessels to bioreactors, in: Paek, K., Murthy, H., Zhong, J. (Eds.), *Production of Biomass and Bioactive Compounds Using Bioreactor Technology*. Dordrecht: Springer, pp. 47–60. https://doi.org/10.1007/978-94-017-9223-3_3

Sugiharto, Kusuma, D.Y., Winarni, D., Bhakti, I.N., Muhsyi, A. H.M., Manuhara, Y.S.W., 2021. Comparison of antioxidant potential of *Gynura procumbens* adventitious root *in vitro* culture and *ex vitro*. *Eco. Env. and Cons.* 27, 1880 - 1884.

Sugiharto, Winarni,D., Wibowo, A.T., Islamatasya, U.,Bhakti, I.N., Nisa, N., Tan, B.C., Manuhara, Y.S.W., 2022. *Gynura procumbens* adventitious root extract altered expression of antioxidant genes and exert hepatoprotective effects against cadmium-induced oxidative stress in mice. *Hayati J. Biosciences*. 29, 479-486. https://doi.org/10.4308/hjb.29.4.479-486

Takanashi, K., Dan, K., Matsuoka, T., Torii, K., 2019. The cancer cell killing effects of *Gynura procumbens*, Toleaf are associated with CD4+ T lymphocyte activation. *Front. Med.* 1, e190004.

Wu, C.H., Murthy, H.N., Hahn, E.J., Paek, K.Y., 2007. Improved production of caffeic acid derivatives in suspension cultures of *Echinacea purpurea* by medium replenishment strategy. *Arch. Pharm. Res.* 30, 945-949. https://doi.org/10.1007/BF02993961

Zhang, Z., Huang, R., 2013. Analysis of malondialdehyde, chlorophyll proline, soluble sugar, and glutathione content in Arabidopsis seedling. *Bio Protoc.* 3, 817-817. https://doi.org/10.21769/BioProtoc.817

Zhao, Y., Guo, W.H., Sun, X.Y., Li, K.H., Liu, K.J., Wang, J., Wang, Y., Tan, X., You, X.L., 2020. A culture system for the stable and high-efficiency proliferation of adventitious roots of *Panax notoginseng* and ginsenoside accumulation. *Ind. Crops. Prod.* 157, 112882. https://doi.org/10.1016/j.indcrop.2020.112882