Sys Rev Pharm 2021; 12(4): 102-112
A multifaceted review journal in the field of pharmacy
E-ISSN 0976-2779 P-ISSN 0975-8453

Research Article

Aeration Volume and Inoculum Density Using in Bioreactor to Optimized Biomass Production and Secondary Metabolites in *Gynura procumbens* (Lour.) Merr. Adventitious Roots Culture

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Article History: *Submitted: 13.04.2021* *Accepted: 20.04.2021* *Published: 27.04.2021*

ABSTRACT

Phenolic compounds and flavonoids are secondary metabolite compositions of the ***Gynura procumbens*** plant. Limited amounts of these secondary metabolites in nature limit their potential as raw material for pharmaceutical drugs. Hence, the optimization of adventitious root culture in vitro should be used as an alternative to producing these compounds. Under optimal culture conditions, the target secondary metabolites can be significantly increased. In this work, we determined the optimum aeration volume and inoculum density on growth and secondary metabolite production of Gynura procumbens adventitious roots in a laboratory-scale bioreactor. The adventitious roots were cultured in a 2 L liquid MS in a 3 L capacity Balloon-Type Bubble Bioreactor (BTBB). The 3 g/L adventitious roots were cultured in 4 variations of aeration volume treatment. The best aeration treatment is 0.15 vvm were used in 4 variations of the inoculum density treatment. The highest biomass (75.38 ± 0.95 g/L), total phenolic contents production (27.98 mg/DW), and flavonoid production rates (256.24 mg/DW) from adventitious roots culture in the BTBB has produced from a treatment combination aeration volume of 0.15 vvm and inoculum density 3 g/L. Accumulation of malondialdehyde and proline in roots in that treatment was lower than other treatments. The four flavonoid compounds that known used as drugs pharmaceutical compound as myricetin, catechin, kaempferol, and quercetin was successfully determined under that treatment. These optimal conditions of aeration volume and inoculum density could be used to mass-produce the desired compounds from Gynura procumbens adventitious root on a pilot scale for industries.

Keywords: *Gynura procumbes*, Bioreactor, Aeration, Inoculum density, Secondary metabolites, Adventitious root

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INTRODUCTION

Gynura procumbens Lour. (Merr.) is a native Indonesian plant that belongs to the Compositae (Asteraceae) family (Hidayat S, 1997). *G. procumbens* has been traditionally consumed as a vegetable in South-East Asian countries like Indonesia, Malaysia, and Thailand (Kaewseejan N, *et al.*, 2015) and used as herbal medicine to treat various diseases, such as fever, rash, hypertension, migraine, constipation, kidney problems, diabetes mellitus, and cancer (Krishnan V, *et al.*, 2015). The root and leaf of *G. procumbens* are often used as a source for drug development, since they contain a variety of bioactive compounds, such as miraculin, (Hew CS, Gam LH, 2011) cerebroside, (Hu JW, *et al.*, 2019) steroid, (Hu JW, *et al.*, 2019) and many forms of flavonoids such as flavonol, flavone, (Kaewseejan N, *et al.*, 2015, You JJ, *et al.*, 2017) flavanone, and phenolic compounds (Manogaran M, *et al.*, 2019, Saiman MZ, *et al.*, 2012). It has been shown that *G. procumbens* does not contain toxic compounds like pyrrolizidine alkaloids compared to other members of the Gynura genus (Saiman MZ, *et al.*, 2012). Other beneficial applications of *G. procumbens* have also been reported. These include cancer treatment, (Jenie RI, *et al.*, 2006, Meiyanto E, *et al.*, 2007, Jenie RI, Meiyanto E, 2009, Nurulita NA, *et al.*, 2012) immunomodulatory booster, (Takanashi K, *et al.*, 2019) vasodilation agent, (Hoe SZ, *et al.*, 2011, Ng HK, *et al.*, 2013) diabetic treatment, (Algariri K, *et al.*, 2013) antivirus, (Jarikasem S, *et al.*, 2013) anti-inflammatory, (Iskander MN, *et al.*, 2002) and antioxidant agent (Krishnan V, *et al.*, 2015, Rosidah MY, *et al.*, 2008). Besides, the extract from *G. procumbens* has been incorporated into antiseptic and cosmetic products, such as hand sanitizer, oral spray, hand soap, skincare cream, skin whitening, anti-aging cream, and moisturizing cream (Xie P, 2007, Xie P, 2007, Xie P, 2007, Xie P, 2007, Park JS, 2018, Choi KH, *et al.*, 2015, Baek SH, *et al.*, 2016).

Despite its beneficial properties, the low amount of these secondary metabolites in nature has limited their applications. Plant cell, tissue, and organ cultures have been widely used to produce beneficial secondary metabolites. With an optimized culture condition, the target secondary metabolites can be significantly enhanced (Baque MA, *et al.*, 2010, Baque MA, *et al.*, 2012). Adequate gas circulation very important for the growth and development of plants in tissue culture, so that aeration need to added in culture system (George EF, 2008) in a culture used airlift bioreactor, aeration very important in three functions, that is keeping the culture conditions aerobic, removing volatile compounds (evaporating), and removing heat from metabolic processes, aeration also functions as a stirring medium to maintain the homogeneity of cell and organ cultures (Murthy HN, *et al.*, 2014) Continuous submersion in liquid media (Ziv M, 2005) and aerobic metabolism (Ho TT, *et al.*, 2017) can induce oxidative stress in the tissue and the formation of Reactive Oxygen Species (ROS). A reactive oxygen species and oxidative stress can activate defense mechanisms through increased activity of antioxidant enzymes, such as Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Reductase (GR) (Esfandiari E, *et al.*, 2007). High aeration also promotes shear stress, so which can promote Phenylalanine

Ammonia-Lyase (PAL) enzyme activities. This enzyme can enhance phenol and flavonoids production and protect cells from oxidative stress (Baque MA, *et al.*, 2013).

Determination of the size or number of plant inoculums in the in vitro system is one of the important steps in using bioreactors (Steingroewer J, *et al.*, 2013). High inoculum density also leads to early competition for nutrition (especially for sugar) acquisition, so this competition could reduce cell multiplication rate and the accumulation of biomass (Adelberg J, Toler J, 2004). Lee EJ reported high initial inoculum can increasing CO2 level in the culture system where is A high level of CO2 in the bioreactor system can inhibit growth and promote cell death (Lee EJ, *et al.*, 2011) High inoculum density might cause excessive shear stress that leads to oxidative stress, lipid peroxidation, and production of MDA, so inoculum density clearly affects the growth and production of active compounds in plant cell cultures (Baque MA, *et al.*, 2014) The high inoculum density stimulates root growth, but in some cases, in adventitious root cultures, there is an inhibition of the production of active compounds, for example, phenols and flavonoids in the root culture of E. angustifolia, (Wu CH, *et al.*, 2006) and eleutheroside compounds B and E in *E. koreanum*, (Lee EJ, *et al.*, 2011).

Previous research in *Gynura procumbens* was achieved lower biomass when cultured using a shaking flask rather than bioreactor culture. Culture using a shaking flask due to lack of oxygen and hyperhydricity due to immersion in a liquid medium because no aeration added (Manuhara YSW, *et al.*, 2017). However, culture using a bioreactor (the previous report using a 1 L capacity bioreactor) allow an aeration volume (0.20 vvm), so that was achieved produced biomass 13-15 folds that of the initial inoculum (Manuhara YSW, *et al.*, 2017, Faizah H, *et al.*, 2018). It has been shown that biomass can be affected by aeration. Some other report for *Gynura procumbens* roots cultures are, sucrose concentration and precursor compounds (phenylalanine and tyrosine), (Noviyanti R, *et al.*, 2017) types of carbon source, (Muthoharoh L, *et al.*, 2019) and elicitation with *S. cerevisia*e extracts and copper (II) sulfate (CuSO4) (Faizah H, *et al.*, 2018) Not yet any report about optimized inoculum density on *Gynura procumbens* adventitious roots culture. Therefore, in *Gynura procumbens* roots culture optimized culture condition in bioreactor not yet well-achieved, mainly about the effect of aeration volume and inoculum density. While the optimized culture condition is indispensable on a larger capacity of a bioreactor which will be applied on an industrial scale.

In this study, we evaluated the effect of different aeration volumes and inoculum densities for optimized the biomass, flavonoid, and phenolic production in *Gynura procumbens* adventitious roots cultured in a 3L large-capacity laboratory-scale bioreactor. The changes of Malondialdehyde (MDA) and proline levels, as well as culture media conditions (pH, sugar level, and electrical conductivity), were also evaluated.

MATERIALS AND METHODS

Plant material

The healthy 1-year-old *Gynura procumbens* (Lour.) Merr. plants obtained from the Purwodadi Botanical Garden, Pasuruan, East Java, Indonesia, were maintained at Airlangga University experimental garden with an ambient temperature of 35ºC, planted in compost medium, watering media 3 times a week, and fertilizing once a week. The young leaves (leaves number 3 to 5) and internodes were sampled, surface disinfected using 10% (v/v) Bayclin® (5.25% active sodium hypochlorite) for 5 min, and rinsed three times with sterile distilled water. The leaf explants were cut into 1-1.5 cm2 /pieces, while the internodes explants were cut into small pieces of 2-3 cm in length. The explants were cultured on Murashige and Skoog (MS) medium (Murashige T, Skoog F, 1962) containing 5 mg/L Indole-3-Butyric Acid (IBA), 30 g/L sucrose, and 6 g/L agar. The media were adjusted to pH 5.8 and autoclaved at 121°C for 15 min. The regenerated adventitious roots (3 weeks-old) were harvested and cut into 1- 2 cm in length. The roots were cultured in 100 mL liquid MS medium with the same composition and maintained at about 25ºC on the dark conditions under constant shaking of 100 rpm on an orbital shaker (IKA-KS130). The explants were subcultured into the original fresh media every 3 weeks.

Adventitious root culture in the bioreactor

The adventitious root cultures amount 3 g/L were transferred into a 3 L airlift Balloon Type-Bubble Bioreactor (BTBB) containing 2 L liquid MS media supplemented with 5 ppm IBA and 30 g/L sucrose for the first experiment various aeration volumes treatment (0.05, 0.10, 0.15, and 0.20 vvm). The best treatment from aeration volume was used for the next experiment various initial inoculum densities (3, 5, 7, and 10 g/L). All cultures were maintained in dark at ± 25ºC for 28 days.

Measurement of biomass

The adventitious roots were harvested, rinsed with water, and gently blotted on a paper towel after 28 days of culture. The fresh and dried roots were weighed using an analytical balance (Shimadzu-LIBROR AEL200). For Dry Weight (DW) measurement, the adventitious roots were dried for 2-3 days at room temperature (30ºC-34ºC). The percentage of DW was calculated by dividing DW with Fresh Weight (FW), while the growth ratio was calculated using the following formula:

Growth ratio=(Final dry weight)-(Initial dry weight)/(Initial dry weight)

Culture media condition measurement

Measurement of media conditions was performed on three stages following are media preparation stage, initiation cultures, and harvest time. The measurement includes: pH was measured using a pH universal indicator (4-7 scale, Merck), electrical conductivity was measured with an hand conductivity meter (Ezodo Cond521) and total sugar was measured using a hand refractometer brix (Atago Master 10T).

Phenolics and flavonoids extraction

About 0.5 g dried in-vitro and ex-vitro (8 months old soil plants) adventitious root powder respectively was macerated using 5 mL (1:10 w/v) 70% methanol to determine total phenolics and total flavonoids. Futhermore, about 25 g dried adventitious root powder was macerated using 250 mL (1:10 w/v) methanol and incubated at 28ºC in a shaker (DAIHAN LABTECH-LSI3016A) under shaking condition of 90 rpm. This step was performed twice. The liquid extract was separated from solid debris using a filter paper before concentrated by evaporation at 28-30ºC. Next, 50 mg of the concentrated extract from in-vitro was dissolved in 10 mL methanol before analyzed using High-Performance Liquid Chromatography (HPLC).

High-Performance Liquid Chromatography (HPLC) analysis

The extract solution (200 µL) was filtered using a 0.45 µm nylon membrane. Next, 20 µL of the filtered extract was injected into an HPLC instrument (Agilent 1100) equipped with an autosampler and DAD detector. The lichrospher 100 RP-18 column (5 µm) 4.6 × 250 mm was used. For myricetin and catechin analysis, acetonitrile eluent and 0.25% acetic acid solution (20:80) were used. The mixture was run at a 1 mL/min flow rate and detected at 340 nm wavelength for myricetin and 280 nm for catechin. For kaempferol and quercetin analysis, acetonitrile eluent and 0.1 % formic acid solution (60:40) was used. The solution was run at a 1 mL/min flow rate and the column temperature was set to 25ºC under UV detection of 299 nm wavelength. The sample iden-

tification was performed by comparing sample and standard solution Retention Time (RT). The concentration of flavonoids was calculated based on the peak area using a linear regression equation from the standard curves of four standard solutions: myricetin, catechin, kaempferol, dan quercetin. The concentration result converted as micrograms per grams DW (µg/g DW).

Total phenolic and flavonoid concentrations measurement

Total phenolic content was measured according to Kaewseejan N (Kaewseejan N, *et al.*, 2015). The absorbance of the sample and methanol (blank) solution was measured using a UV spectrophotometer (BOECO S-22, Germany) at 765 nm. The concentration of total phenolic compounds was determined by regression analysis based on the standard curve obtained from the absorbance rate of standard phenolic acid and expressed as mg GEA (gallic acid equivalent) per gram of DW. Total flavonoid content was measured using the modified method of Kaewseejan N (Kaewseejan N, *et al.*, 2015) About 250 µL of the methanol extract was mixed with 125 mL distilled water and 75 µL of 5% (w/v) sodium nitrite. The mixture was incubated at room temperature for 5 min before adding 150 µL of 1% (w/v) aluminium chloride. After 5 min of incubation, 150 µL of 1 M sodium hydroxide was added and topped up with distilled water to a final volume of 2.5 mL. The absorbance of the sample and methanol (blank) solution was measured using a UV spectrophotometer (BOECO S-22, Germany) at 510 nm. The concentration of total flavonoids was determined using regression analysis based on the standard curve obtained from the absorbance rate of standards: quercetin and kaempferol and expressed as milligram equivalent of kaempferol and quercetin (Eq. mg kaempferol/g DW and mg quercetin/g DW, respectively).

Malondialdehyde (MDA) concentration measurement

MDA was measured according to Zhang and Huang (Zhang Z, Huang R, 2013). About 0.5 g of fresh adventitious roots were homogenized into fine powder in the presence of liquid nitrogen before adding 4.5 mL of 1% sulfuric acid. After centrifuged at 3,000 rpm for 10 min, 0.5 mL supernatant was mixed with 0.5 mL of 20% thiobarbituric acid and 2 mL of thiobarbituric acid + thiobarbiturate solution. The mixture was incubated at 95°C in a water bath for 60 min and immediately cooled on ice. The mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was measured at 523 nm using a UV spectrophotometer (BOECO S-22, Germany). The concentration of MDA was determined using regression analysis based on the standard curve obtained from MDA standard solution (0-40 nmol). The results were presented as nmol/0.5 g FW.

Proline concentration measurement

Proline content was measured according to Bates LS (Bates LS, *et al.*, 1973). First, 0.5 g of adventitious root powder was mixed with 4.5 mL of 3% sulfosalicylic acid before centrifuged at 3,000 rpm for 10 min. Next, 0.5 mL of the collected supernatant was mixed with 1 mL ninhydrin acid and 1 mL glacial acetic acid. The mixture was incubated at 95°C in a water bath for 60 min and immediately cooled on ice for 30 min. A total of 1.5 mL of toluene was added into the solution and mixed thoroughly by vortex for 1 min. Separated Chromophore from toluene was collected using a pipette and its absorbance at 520 nm was measured using a UV spectrophotometer (BOECO S-22, Germany). The concentration of proline was calculated based on the standard curve made using L-proline standard solution (0-300 µM). The results were presented as µM /0.5 g FW.

Statistical analysis

Data with 2 replicates such as biomass, pH, total sucrose content, the electrical conductivity of culture media, and HPLC result were analyzed by described quantitatively. While data with 3 replicates such as total phenolic and flavonoid data, and malondialdehyde and proline measurement data were analyzed using one-way analysis of variance ($p<0.05$) followed by Duncan's multiple range test (DMRT) at 5% level using SPSS software.

RESULTS AND DISCUSSION

Aeration volume enhanced the biomass and total phenolic and flavonoid contents

The effect of aeration volume on the growth of the adventitious roots *Gynura procumbens* in a bioreactor was evaluated through biomass and growth ratio measurement. The growth result of adventitious roots indicated at the aeration volume 0.15 vvm and 0.20 vvm was increased root fresh and dry weight (Table 1 and Figure 1). Although the biomass and growth ratio were slightly declined at 0.20 vvm, that's indicating 0.15 vvm is the optimum aeration volume for root growth (Table 1).

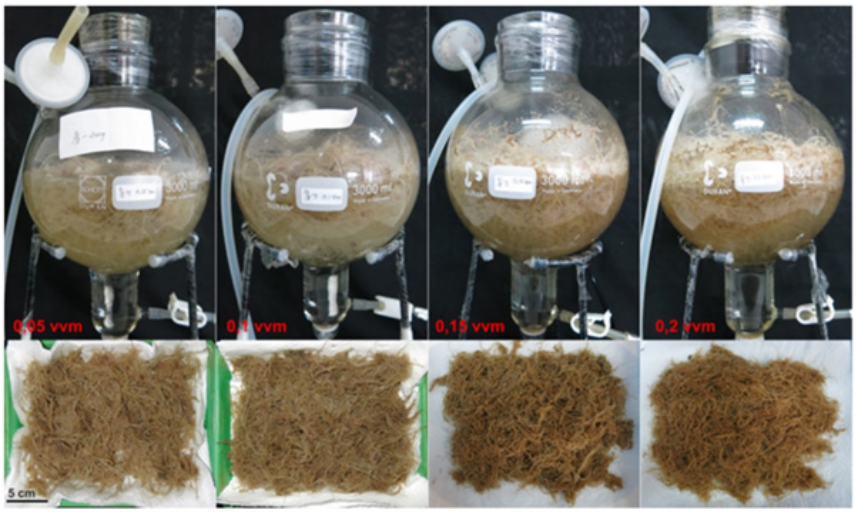

Figure 1: Adventitious root cultures of *Gynura procumbens* in a 3 L bioreactor for 28 days using various aeration volumes

Table 1: The effect of various aeration volumes on the biomass of *Gynura procumbens* adventitious root cultured in 3 L bioreactor (with 2 L media) for 28 days

Aerations (vvm)	Fresh weight (g/L)	Dry weight (g/L)	Dry weight (%)	Growth ratio
0.05	31.32 ± 11.69	1.38 ± 0.60	4.39	12.74
0.1	41.69 ± 14.47	1.97 ± 0.88	4.72	18.69
0.15	75.38 ± 0.95	4.01 ± 0.21	5.32	39.1
0.2	62.43 ± 17.85	3.70 ± 0.35	5.92	35.95
± SD (n=2)				

An aeration volume might be affecting on air agitation and oxygen (O2) penetration into the media, thus increasing dissolved oxygen level in the culture media (Ahmed S, *et al.*, 2008). Increased dissolved oxygen has positive effect on cultured cells and root growth, as treatment used high aeration volume at 0.15 vvm and 0.2 vvm affected to increase growth ratio *Gynura procumbens* adventitious root cultured in 3 L bioreactor, especially at 0.15 vvm. That's phenomena has been reported before in the cell suspension culture of *M. citrifolia* (Ahmed S, *et al.*, 2008) and adventitious root culture of *E. koreanum* Nakai (Lee EJ, *et al.*, 2011) and A. membranaceus (Wu SQ, *et al.*, 2011). Compared to *G. procumbens* adventitious root cultured in 1 L bioreactor with aeration volume 0,2 vvm (Faizah H, *et al.*, 2018), we found 20% and 8% increase in growth ratio when the adventitious roots have cultured in 3 L bioreactor with 0.15 vvm and 0.2 vvm aeration volume, respectively.

High aeration volume at 0.2 vvm or above can negatively affect explant growth because it leads to excessive shear stress, subsequent cell wall thickening, and inhibition of explant growth. Similar negative effect of high aeration volume (≥ 0.2 vvm) on growth was also reported for adventitious culture of *E. koreanum* Nakai (Wu SQ, *et al.*, 2011). On the other hand, low aeration volume (0.05 vvm) can also inhibit growth, because it can increase dissolved carbon dioxide (CO2) level in the media that negatively affect respiration and metabolism processes. Accumulation of CO2 due to low aeration volume was previously reported by Lee EJ (Lee EJ, *et al.*, 2011).

In this work, we also found that the use of high aeration volume in large-capacity bioreactor (3 L) can increase contamination rate in the media, a problem that not reported and occurred before in smaller-capacity (1 L) bioreactor (Faizah H, *et al.*, 2018, Saadah IN, *et al.*, 2019). This was caused by rapid air flow and formation of air bubbles on the surface of the media. Accumulation of air bubbles lead to condensation of the air filter that can increase contamination rate in the media. A Similar phenomenon has reported by Wu SQ for adventitious root culture of *Astragalus membranaceus* (Wu SQ, *et al.*, 2011). Using aeration in the bioreactor culture system also can cause water evaporating on culture media. We found decreased media volume on harvest periods on all aeration treatment. These phenomena have been confirmed by Murthy in them report (Murthy HN, *et al.*, 2014).

The phenolic and flavonoid content is also influenced by the volume of aeration. The highest phenolic production rates and flavonoid production rates were found in adventitious roots with an aeration volume of 0.15 vvm. It should be noted that the total flavonoids and phenolic compounds from 4 weeks of *G. procumbens* adventitious root from the bioreactor culture were almost closed to those of 8 months old *G. procumbens* field plants (Table 2).

Mild shear stress caused by 0.15 vvm volume might induce higher production of secondary metabolites but not too strong to caused growth inhibition, therefore optimum growth and metabolites production can be achieved at the same time (Tables 1 and 2). In accordance, similar results were reported for suspension culture of *M. citrifolia* and *G. uralensis* (Gao WY, *et al.*, 2014) and adventitious root culture of *E. koreanum* Nakai (Lee EJ, *et al.*, 2011). We found that's low aeration volume (0.05 vvm) lead to the highest accumulation of phenolic, but not flavonoid compounds. Indicating that stress caused by high level of dissolved CO2 in the media might induced higher production of phenolic compounds. Positive correlation between low aeration volume (<0.1 vvm) and phenol production have been reported in *E. Purpurea* root culture (Murthy HN, *et al.*, 2014). On the other hand, shear stress due to high aeration volume (≥ 0.2 vvm) was reported to induce higher flavonoid production in *H. perforatum* (Cui XH, *et al.*, 2014) and *M. citrifolia*, (Baque MA, *et al.*, 2014) that suggesting that phenol and flavonoid production has promoted by different types of stress and mechanism.

Table 2: The effect of various aeration volumes on total phenolics, total flavonoids, phenolics production rate, and flavonoids production rate content of *Gynura procumbens* adventitious root cultured in 3 L bioreactor (with 2 L media) for 28 days

Aeration (vvm)	Total Phenolic (mg/g DW)	Phenolics Production rate* (mg/DW)	Flavonoids (mg/g DW)		Total (mg/g DW)	Flavonoids production rate* (mg/DW)
			Eq. Kaempferol	Eq. Quercetin		
0.05	7.14 ± 0.14	9.85	43.42 ± 0.68	11.92 ± 0.20	55.34	76.39
0.1	6.04 ± 0.14	11.89	39.40 ± 0.56	10.72 ± 0.16	50.12	98.73
0.15	6.98 ± 0.16	27.98	50.00 ± 0.60	13.90 ± 0.18	63.9	256.24
0.2	6.40 ± 0.12	23.68	40.30 ± 0.90	10.98 ± 0.26	51.28	189.73
Ex vitro roots	10.08 ± 0.07	-	54.30 ± 0.46	15.20 ± 0.14	69.5	-
± SD (n=3), ANOVA (p<0.05), DMRT significance at 5% level						
*Production rate: total content (mg/g DW) × Dry weight (g/L)						
**Total flavonoid content: eq. kaempferol + eq. quercetin						

Initial inoculum density altered the biomass, total phenolic and total flavonoid contents

The optimal aeration volume (0.15 vvm) was used to evaluate the effect of various initial inoculum densities on plant growth and its phenolic and flavonoid contents. We found that adventitious roots cultured in a bioreactor with an initial inoculum density of 3 g/L (samely to the first table result) remain higher in produced the fresh and dry weight, and growth ratio than others initial inoculum density treatment. The biomass was decreased with an increasing amount of initial inoculum (Table 3 and Figure 2). Besides, the initial inoculum density of 3 g/L was also remaining induced a significantly higher accumulation of total phenolic and flavonoid content than others initial inoculum density treatment (Table 4).

The results suggest that initial inoculum density is one of the main determinants for large-scale culture productivity, similar finding was reported for adventitious root culture of *P. multiflorum*, (Ho TT, *et al.*, 2017) *P. ginseng*, (Hahn EJ, *et al.*, 2003) and *E. purpurea* (Jeong JA, *et al.*, 2009). In this work we found that the optimum initial inoculum density for *G. procumbens* adventitious culture is 3 g/L. There are several factors that affecting explant growth in the media, among them are CO2 level and availability of nutrition. We guessed the high inoculum might have occurred limitation of nutrition at culture periods, when the biomass reaching at middle culture periods and occurs nutrition depletion also increase high level of CO2, so the root's did'n growing-well and the biomass has been reduced at the end of culture.

Table 3: The effect of various initial inoculum densities on the biomass of *Gynura procumbens* adventitious root cultured in 3 L bioreactor (with 2 L media) for 28 days

Inoculums (g/L)	Fresh weight (g/L)	Dry weight (g/L)	% dry weight	Growth ratio
3	75.38 ± 0.95	4.01 ± 0.21	5.32	39.1
5	58.38 ± 9.72	2.66 ± 0.73	4.55	6.08
7	60.80 ± 12.80	2.69 ± 0.69	4.42	4.27
10	62.13 ± 12.20	2.93 ± 1.22	4.71	2.87
± SD (n=2)				

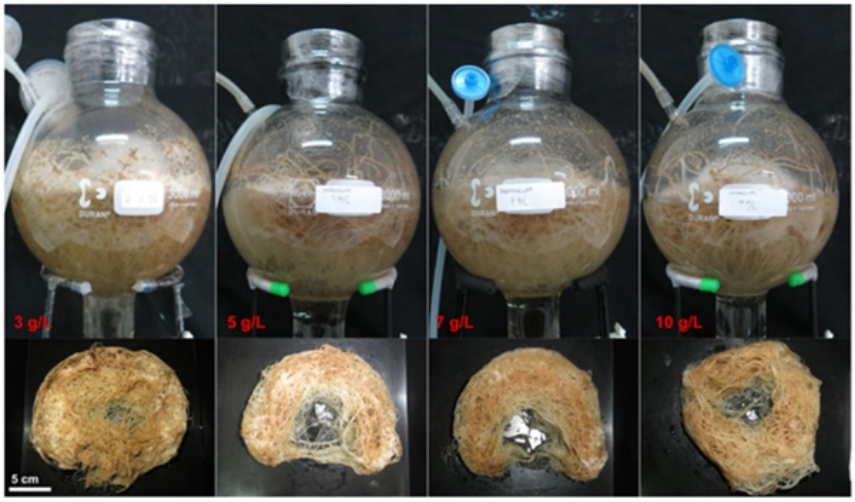

Figure 2: Adventitious root culture of Gynura procumbens in a 3 L bioreactor for 28 days, using various initial inoculum densities

Table 4: The effect of various initial inoculum density on total phenolics, total flavonoids, phenolics production rate, and flavonoids production rate content of *Gynura procumbens* adventitious root cultured in 3 L bioreactor (with 2 L media) for 28 days

Inoculum density (g/L)	Total Phenolic (mg/g DW)	*Phenolics production rate (mg/DW)	Flavonoids (mg/g DW)		**Total flavonoids (mg/g DW)	*Flavonoids production rate (mg/DW)
			Eq. Kaempferol	Eq. Quercetin		
3	6.98 ± 0.16	27.98	50.00 ± 0.58	13.90 ± 0.18	63.9	256.24
5	2.94 ± 0.06	7.82	22.88 ± 0.44	5.76 ± 0.13	28.64	76.18
7	2.76 ± 0.04	7.42	22.44 ± 0.22	5.63 ± 0.06	28.07	75.5
10	5.28 ± 0.20	12.62	46.22 ± 0.22	12.80 ± 0.06	59.02	172.92
Ex vitro roots	10.08 ± 0.07	-	54.30 ± 0.46	15.20 ± 0.14	69.5	-
± SD (n=3), ANOVA ($p<0.05$), DMRT significance at 5% level						
*Production rate : total content (mg/g DW) × Dry weight (g/L)						
**Total flavonoid content : eq. kaempferol + eq. quercetin						

High inoculum density also leads to early competition for nutrition acquisition, it could reduce cell's multiplication rate and the accumulation of biomass, like's occurred on *Alocasia macrorrhizos* and *Colocasia esculenta* shoot cultures (Adelberg J, Toler J, 2004). Lee EJ (Lee EJ, *et al.*, 2011) also reported, high level of carbon dioxide (CO2) in the bioreactor system can inhibit growth and promote cell death, as occured on adventitious root culture of *E. koreanum* Nakai. The highest inoculum density treatment in this study (10 g/L) showed a fairly high yield of biomass and secondary metabolite production. increased root density allows optimal root growth to be achieved in a short time, but also results in faster root mortality. this phenomenon may lead to changes in the total productivity of phenolics and flavonoids.

Aeration volumes and inoculum densities increased concentrations of Malondialdehyde (MDA)

Lipid peroxidation occurs due to cellular oxidative stress triggered by biotic or abiotic stresses (Davey MW, *et al.*, 2015). The final product of lipid peroxidation is Malondialdehyde (MDA), wherein high accumulation of MDA has been regarded as an indicator of membrane cellular damage (Baque MA, *et al.*, 2014). We found that low aeration volume treatment (0.05-0.10 vvm) induced a significantly higher level of MDA in adventitious roots than high aeration volume treatment (0.15-0.20 vvm) and adventitious roots from the field (ex-vitro root) (Figure 3a). The highest concentration of MDA concentration was found in adventitious roots cultured in a bioreactor with 0.10 vvm aeration volume treatment.

The contents of Malondialdehyde (MDA) were increased in line with a high amount of initial inoculum. The concentration of MDA in adventitious roots from bioreactor culture was significantly higher than adventitious roots from the field (ex vitro root), excepted 3 g/L inoculum density treatment was lowest than others (Figure 3b).

In this work, low aeration volume (0.05 vvm) might cause submersion of explants in the liquid media, so promote an increased level of dissolved CO2 in culture media because of aerobic metabolism activity (Lee EJ, *et al.*, 2011). This condition leads to reduced oxygen absorption and stress due to hypoxia, that in turn promotes the production of Nitric Oxide (NO) and Reactive Oxygen Species (ROS) (Wany A, Gupta KJ, 2018). It was reported that the use of high aeration volume can also induce the accumulation of hydrogen peroxide (H2O2) and ROS formation (Baque MA, *et al.*, 2013). We found that MDA was also produced in culture with high aeration volume, but at significantly lower level compared to culture with low aeration volume, indicating lower H2O2 and ROS production in culture with high aeration volume (Figure 2a). When cells exposed to oxidative stress, they could activate defense mechanism through increased activity of antioxidant enzymes, such as Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Reductase (GR). Increased activity of these enzymes that can reduce ROS formation (Esfandiari E, *et al.*, 2007). Shear stress from the use of high aeration volume can also promote Phenylalanine Ammonia Lyase (PAL) enzyme activities. This enzyme can enhance phenol and flavonoids production and protect cells from oxidative stress (Baque MA, *et al.*, 2013). These defense mechanisms might contribute to lower MDA level and higher phenol and flavonoid contents in culture with high aeration volume.

High inoculum density might cause excessive shear stress that leads to oxidative stress, lipid peroxidation, and production of MDA. Interestingly, opposite result was reported for adventitious root culture of *H. perforatum*, where increase in initial inoculum density leads to reduced level of MDA accumulation in the culture (Cui XH, *et al.*, 2014). It was proposed that in *H. perforatum*, high inoculum density could promote the activity of antioxidant enzymes leading to reduction of ROS and MDA production (Cui XH, *et al.*, 2014). These results suggest species-specific effect of initial inoculum density on the culture.

Aeration volumes and initial inoculum density affected concentrations of proline

The highest concentration proline concentration was found in adventitious roots cultured in a bioreactor with 0.10 vvm aeration volume treatment. whereas aeration 0.05 vvm produced the lowest of proline content (Figure 4a). All aeration volume treatments induce higher proline concentration in adventitious roots than adventitious roots from the field (ex vitro root). Likewise for the proline concentration affected by inoculum density in adventitious roots from bioreactor culture was significantly higher than adventitious roots from the field (ex vitro root) (Figure 4b).

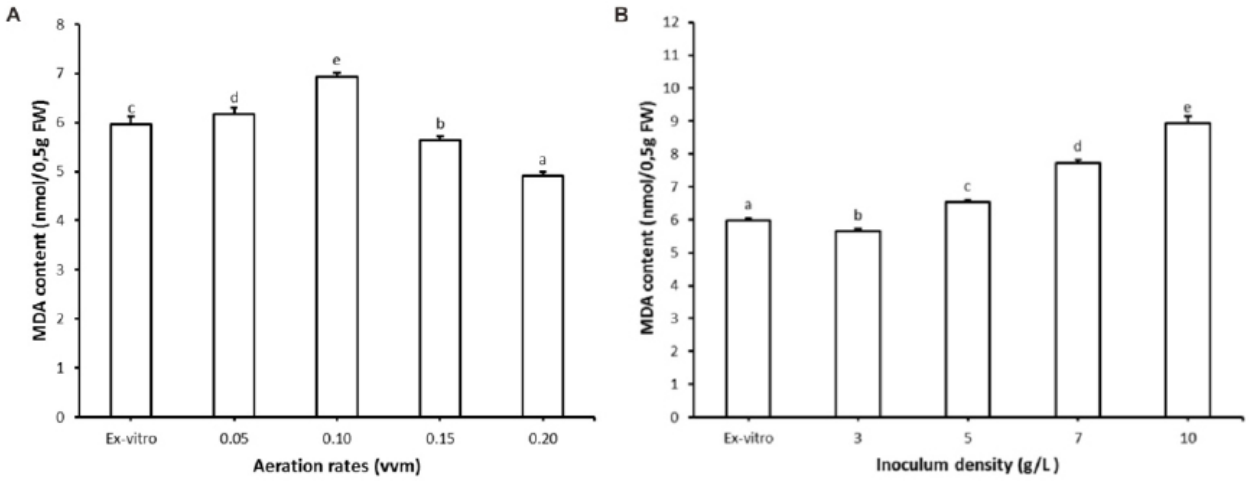

Figure 3: The concentrations of Malondialdehyde (MDA) from *Gynura procumbens* adventitious roots harvested from in vitro roots with (A) various aeration volumes treatment and (B) various initial inoculum treatment compare to ex vitro plant. The bar represents SD (n=3), DMRT significance at 5% level

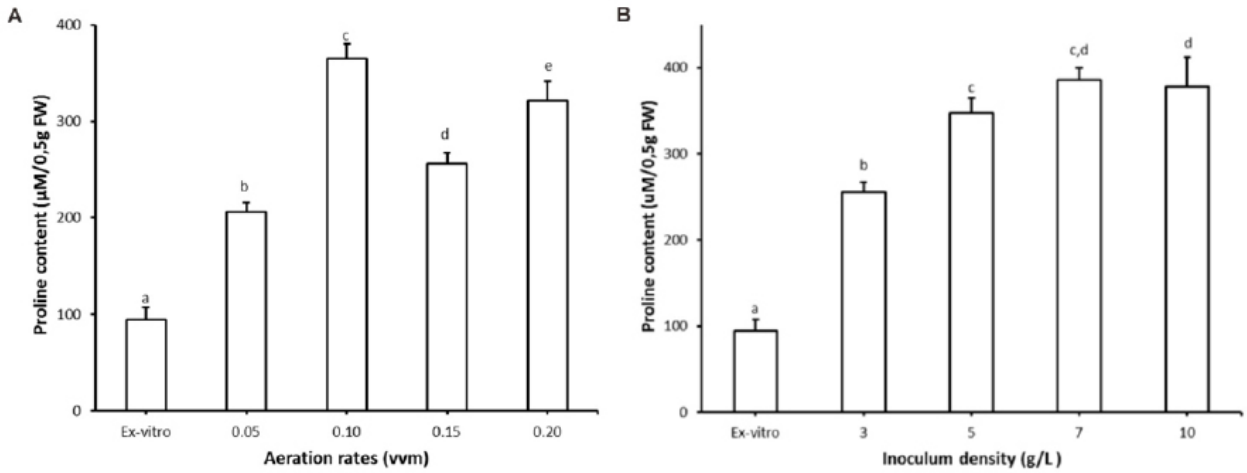

Figure 4: The concentrations of proline from *Gynura procumbens* adventitious roots harvested from in vitro roots with (A) various aeration volumes treatment and (B) various initial inoculum treatment compare to ex vitro plant. The bar represents SD (n=3), DMRT significance at 5% level

In this work, proline accumulation in adventitious root tissue might be a response to a submerged condition in liquid culture media. compared with non-submerged roots (ex vitro roots), proline concentration in all treatments in bioreactor culture ware increased (Figure 2b and Figure 5b). This phenomenon is similar to Barickman TC report, wherein cucumber plants subjected to waterlogging stress for 10 days accumulated significantly higher amounts of proline compared to the non-waterlogged plants (Barickman TC, *et al.*, 2019). The accumulation of proline as an osmolyte could have contributed to maintaining plant water status and hydraulic conductivity during waterlogging stress. This could also emphasize the role of proline in membrane stabilization and maintaining cytosolic pH levels, along with other metabolic functions (Barickman TC, *et al.*, 2019). Proline function in membrane stabilization might occur during high concentration of sugar and ionic minerals formed in the culture media because water evaporated during treatment on high aeration (0.20 vvm). Besides evaporating, water absorption by high inoculum densities of explants might cause low water potential in the media (Thorpe T, *et al.*, 2008). In accordance, culture with high inoculum density produced an adventitious root with a low dry weight percentage (Tables 3 and 4), thas is might indicates high water content in the adventitious roots tissues. Excessive water absorption by explants might leads to reduced water potential in the media that in turn affecting ion and sugar absorption for root growth and development.

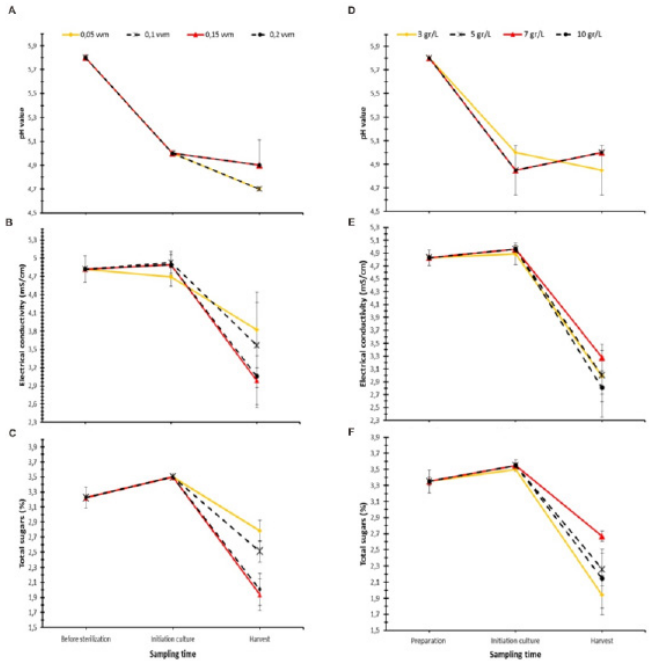

Figure 5: Aeration volumes and initial inoculum density change conditions level of culture media. Figure A-C are representatives from various aeration volumes treatment and Figure D-E are representatives from inoculum density treatment

Aeration volumes and initial inoculum density changes conditions level of culture media

The measurement of pH, electrical conductivity, and total sugar level content of the culture media was performed at three stages following are media preparation stage, initiation cultures, and harvest time. We found that aeration volume slightly affected on pH culture media. The pH slightly decreases from initiation culture to harvest time at aeration volume >0.05 vvm, but more decreased at low aeration volume (0.05 vvm) (Figure 5a). However, the electrical conductivity and total sugar content were extremely decreased at the harvest period (Figures 5b-5c).

The pH of media with an initial inoculum density of 3 g/L was decreased at the end of the culture period, while 5, 7, and 10 g/L initial inoculum densities increased the pH of media (Figure 5d). Similar to aeration treatment, the electrical conductivity and total sugar content were extremely decreased for all inoculum densities treatments (Figures 5e-5f).

Changes in pH could affect minerals and nutrition absorption by adventitious roots (Ahmad N, *et al.*, 2018). It has reported that H+ ion increase in the media can decrease pH and promotes ammonium assimilation in the cultured organs (Manuhara YSW, *et al.*, 2015). On the other hand, when H+ ion decreases, the pH of the media will increase and promotes nitrate assimilation into the organs (Ahmad N, *et al.*, 2018). In this work, pH 5 seems to be the optimum condition for *G. procumbens* adventitious root growth in 3 L bioreactor with 0.15 vvm aeration volume. Previous works reported different optimum pH for other species, for example *Panax ginseng* (Baque MA, *et al.*, 2013) dan *Stevia rebaudiana* (Ahmad N, *et al.*, 2018) adventitious growing optimally at pH 6-6.5.

The conductivity value of the media is a good predictor of biomass production rates (Saiman MZ, *et al.*, 2012). High absorption of ions indicates high metabolism and growth rates. The use of aeration volume 0.15 vvm seems to promote ions absorption and cellular metabolism through sufficient supply of oxygen. Similar results have been reported for adventitious root culture of *E. purpurea* (Murthy HN, *et al.*, 2014), *H. perforatum* (Cui XH, *et al.*, 2014) and *Talinum paniculatum* (Manuhara YSW, *et al.*, 2015).

Sugar is the main source of nutrition required for growth and metabolism for the plant organ cultures. It can also affect ion minerals absorption and assimilation (Wu CH, *et al.*, 2006). The decrease of sugar content in the media is expected along with the increase of adventitious root growth and biomass (Figure 3c). The drastic reduction in total sugar concentration also showed the highest biomass recovery in *G. procumbens* adventitious root culture in temporary immersion bioreactor (Kusuma DY, *et al.*, 2016). In the inoculum density treatment, it appears that the high density root culture did not produce optimal biomass. It is suspected that the absorption of ions and sugars in this treatment was not optimal, so that the ion residue and total sugar in the measured media were in higher concentrations (Figures 6c-6d). Sugar competitions might be the limiting factor for explants growths and high initial inoculum density might promote such competition (Adelberg J, Toler J, 2004).

Identification and concentration of flavonoid compounds from G. procumbens adventitious root extract

In this study, we determined the amount of 4 selected flavonoids, namely myricetin, catechin, quercetin, and kaempferol, for adventitious roots with an initial inoculum of 3 g/L and cultured under 0.15 vvm aeration volume. Among the flavonoids tested, kaempferol and myricetin were the highest amount of flavonoids detected (Table 5 and Figures 6A and 6B).

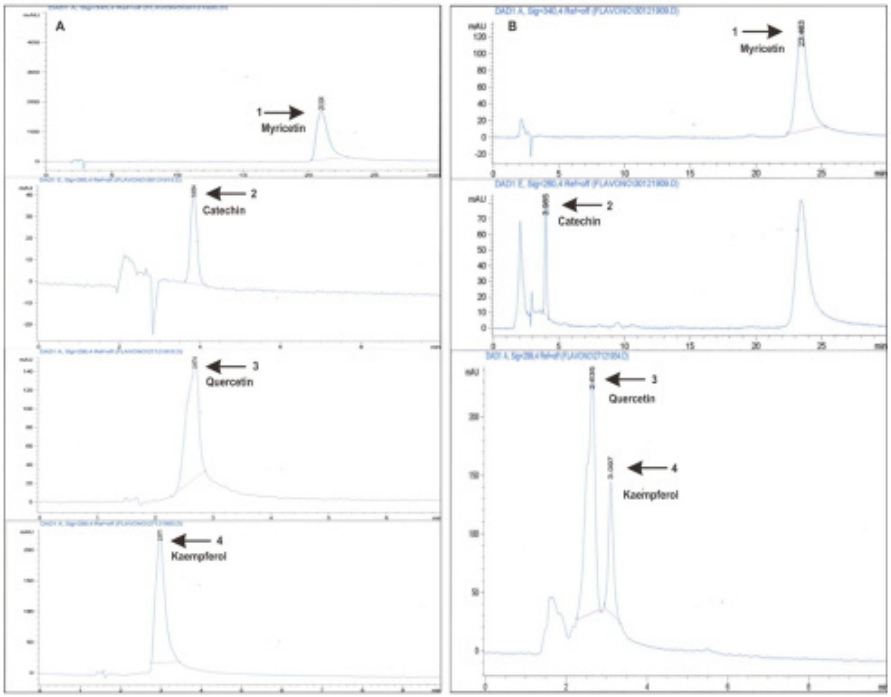

Figure 6: Determined of flavonoid compounds by HPLC. The profiles of (A) standard solution of flavonoid compounds, and (B) flavonoids compounds from adventitious roots *G. procumbens* samples (1) myricetin, (2) catechin, (3) quercetin, and (4) kaempferol

Similar results were reported for *G. procumbens* leaf extract, kaempferol and myricetin were also found to be the main metabolites produced in the leaf (Kaewseejan N, *et al.*, 2015). In addition, kaempferol and myricetin were also found at high concentration in the whole plant's water extract of G. procumens Kaempferol and myricetin are bioactive compounds with potential medicinal properties. Previous works reported the use of kaempferol for cancer, arteriosclerosis, and cardiovascular treatment, it also shown to have antioxidant and anti-inflammation activities (Kaewseejan N, *et al.*, 2015). While myricetin was reported to have anti-viral and anti-diabetic properties (Kaewseejan N, *et al.*, 2015). Our HPLC analysis suggests that the adventitious root of *G. procumbens* contains a variety of beneficial bioactive compounds that can be used as pharmaceutical ingredients. Therefore, continuous optimization for biomass and metabolites production is required, especially for phenolic and flavonoid compounds.

CONCLUSION

Overall, the aeration volume and initial inoculum density of adventitious roots of *G. procumbens* in a 3 L BTBB have been shown to significantly influence the biomass and the production of secondary metabolites. The aeration volume of 0.15 vvm increased the biomass of adventitious roots and the contents of total phenolic and flavonoids while reduced accumulation of MDA and proline. When cultured with an initial inoculum density of 3 g/L, the biomass and secondary metabolite production of adventitious roots were increased, whereas the levels of MDA and proline were reduced. Both aeration volume and initial inoculum density did not affect the pH of the media. Under these optimal conditions, 4 selected flavonoids, namely myricetin, catechin, quercetin, dan kaempferol, were successfully determined.

ACKNOWLEDGMENTS

This research was funded by Mandatory Research of Universitas Airlangga, Indonesia, grant number 331/UN3.14/LT/2019.

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