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A novel pilot bioreactor for scaling up biomass and bioactive compounds on *Gynura procumbens* adventitious root culture

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ABSTRACT Bioreactors for adventitious root culture have been developed to obtain biomass and plant bioactive compounds in large quantities. These technologies provide a great opportunity to produce biomass and bioactive compounds more quickly from *Gynura procumbens* compared to conventional plant cultivation systems. In previous studies, biomass and bioactive compounds of *G. procumbens* adventitious roots were successfully increased using a small-scale bioreactor. In this study, a pilot bioreactor the capacity of 19 L polycarbonate gallon was successfully developed. This bioreactor can be sterilized under the pressure of 0.18 MPa for approximately 60 min. While the bioreactor could not be sterilized when the pressure was less than 0.18 MPa damage may have occurred to the bioreactor vessel at pressures exceeding 0.18 MPa. The results of the chemical grade test as root culture media showed that MS-Tek provided an optimal root biomass compared to MS-PA after a 35-day of the culture period. In addition, the productivity of the total phenolics and flavonoids of adventitious root in MS-PA was higher than in MS-Tek. This novel pilot bioreactor is suitable for *G. procumbens* adventitious root culture, and the technical-grade chemicals are suitable for improving root biomass production.

KEYWORDS Adventitious root; Bioactive compound; Chemical grade; *G. procumbens*; Pilot bioreactor

1. Introduction

Exploration of plant secondary metabolites through adventitious root culture is one of the focuses of commercial industry improvement. Many studies have reported success in increasing adventitious root culture production at the large-scale level for commercial production (Murthy et al. 2019). The products of adventitious root cultures are guaranteed to be safe as they generally contain non-toxic and non-carcinogenic compounds (Murthy et al. 2015). Therefore, the mass production of adventitious roots of *Panax ginseng* and *Echinacea purpurea*, together with their bioactive compounds, have been approved by the Korean Food and Drug Administration (KFDA) and recognized by the United States Food Drug Administration (US FDA) (Murthy and Paek 2014; Mai et al. 2022).

The achievement of industrial-scale production cannot be separated from the intensive development of bioreactors to optimize the production of adventitious roots *in vitro*. Airlift bioreactors are ideal for organ cultures such as hair and adventitious roots, since the bioreactor design is equipped with a sparger to evenly distribute air (Murthy et al. 2016). The design and construction of the airlift-type bioreactor are ideal for scaling up adventitious root cultures since they provide very little shear stress on the plant organ culture by adjusting the capacity and configuration of supporting factors that are important for plant growth development (Murthy et al. 2016).

Based on Saiman et al. (2012), adventitious root culture in *Gynura procumbens* has been developed for the metabolomic study. *G. procumbens* is a medicinal plant that commonly grows in tropical Asian countries, particularly in Indonesia, and contains potential bioactive compounds such as antioxidants (Kaewseejan et al. 2015). The results of the selection of various plant organs by Krishnan et al. (2015) showed that bioactive compounds with potential antioxidants were synthesized more in the root of *G. procumbens* than in other plant organs. In the previous studies, it has been reported that higher antioxidant content and hepatoprotective activity were accumulated in *G. procumbens* root extract cultivated *ex vitro* and *in vitro* (Sugiharto et al. 2021, 2022). Based on its potential, establishment the method and scale-up production process of adventitious roots in the laboratory are considered to produce the bioactive compounds for local industrial raw material needs.

Study on the adventitious root culture of *G. procumbens* has reached the stage of culture optimization, using

several types of bioreactors (Manuhara et al. 2017). Optimized culture using an airlift model balloon-type bubble bioreactor (BTBB) with a capacity of 1 L has been reported in several studies to increase the productivity of the biomass and flavonoid compounds of adventitious roots of *G. procumbens* (Faizah et al. 2018; Muthoharoh et al. 2019; Manuhara et al. 2020). A recent study has reported that the adventitious root production capacity of biomass and flavonoid compounds of *G. procumbens* could be improved using a 3L capacity BTBB, with optimization of the aeration and inoculum density (Kusuma et al. 2021).

Based on the previous report, to enhance the biomass and bioactive compounds of *G. procumbens* adventitious root, an airlift bioreactor with a larger capacity can use on the culture (Kusuma et al. 2021). Since the raw materials for producing bioreactors are expensive, it is necessary to develop large bioreactors at an affordable cost. In addition, a large volume of culture media is required for root culture in large bioreactors. Therefore, appropriate optimization and affordable chemical reagents to prepare the culture media are needed to establish the culture method. Hence, it is very important to develop a suitable pilot bioreactor and low-cost culture media in laboratory-scale production for scaling up the adventitious roots of *G. procumbens*.

2. Materials and Methods

2.1. Development of a pilot plant bioreactor with a capacity of 19 L

The design and configuration of airlift bioreactor pilot used for adventitious root culture of G. procumbens was adapted to the balloon-type bubble bioreactor (BTBB) working system (Paek et al. 2001). A pilot bioreactor was made by modifying a capacity of 19 L polycarbonate.

2.2. Bioreactor preparation and adventitious root inoculation

The bioreactor and its compartments were sterilized in two stages, first in an empty condition and then with 10 L of culture media. The second sterilization stage was performed three days after the first sterilization. The bioreactor was sterilized by the method using an autoclave at different pressures, i.e., 0.12, 0.15, 0.18 and 0.20 MPa, and contamination was observed after the second stage of sterilization for 20 days.

The 30 g of root fresh weight was used for inoculum in the bioreactor culture and the method of culture was used from Kusuma et al. (2021). Murashige and Skoog (MS) media was added to 30 g of sugar and 5 mg/L of Indole-3-butyric acid (IBA) (Merck, Germany). The macronutrients of MS media (NH_4NO_3, KNO_3, $MgSO_4$, $CaCl_2$, and KH_2PO_4) from different grades (based on the purity level according to price and product label) were used in the media composition; namely, full-strength MS media with technical-grade chemicals (MS-Tek) (Pudak Scientific and Smart Lab, Indonesia), and full-strength MS with analytical-grade chemicals (MS-PA) (Merck, Germany). Cultures were incubated in a dark room at room temperature (±26 °C) for 35 days (Faizah et al. 2018).

2.3. Determination of adventitious root biomass and bioactive compounds

Determination of the root biomass was determination based on Kusuma et al. (2021). The adventitious root biomass in fresh weight and dry weight conditions is represented by FW and DW respectively. The determination of the growth ratio (GR) was calculated using the following formula:

$$\mathrm{GR} = \frac{DW2 - DW1}{DW1} \quad (1)$$

DW1 : dry weight of root inoculum
DW2 : dry weight of roots harvested

Determination of the bioactive compound method was modified based on Kaewseejan et al. (2015) using a UV-vis spectrophotometer (Thermo BOECO S-22). Total phenolic content was determined as follows. 200 µL of sample extract was added to 1 mL of Folin–Ciocalteau reagent (Merck, Darmstadt, Germany) (1:10 with distilled water) and left for 5 min. 0.8 mL of 7.5% sodium carbonate (Na_2CO_3) (Merck, Darmstadt, Germany) solution was then added and allowed to stand for 30 min at room temperature. The blank solution was 70% methanol, which was treated in the same way as the test sample. Gallic acid was used for the standard solution to determine total phenolic content at absorbance 765 nm, and the results were represented as mg/L GAE (gallic acid equivalent) per g root sample.

Total flavonoid content was determined as follows. Two hundred and fifty µL of the sample extract was mixed with 125 mL distilled water and 75 µL of 5% (w/v) sodium nitrite (Merck, Darmstadt, Germany). The mixture was incubated at room temperature for 5 min, then 150 µL of 1% (w/v) aluminium chloride (Merck, Darmstadt, Germany) was added. After 5 min of incubation, 150 µL of 1 M sodium hydroxide (Merck, Darmstadt, Germany) was added and topped up with distilled water to a final volume of 2.5 mL. The blank solution was 70% methanol, which was treated in the same way as the test sample. Kaempferol was used for the standard solution to determine the total flavonoid content at absorbance 510 nm, and the results were represented as mg/L KE (kaempferol equivalent) per g root sample.

The productivity of the bioactive compound was determination based on Kusuma et al. (2021). The productivity of phenolic compounds is the multiple of total phenolic content and total root dry weight, with the results represented as mg/L GAE per bioreactor. In addition, the productivity of the flavonoid compounds is the multiple of total flavonoid content and total root dry weight, with the results represented as mg/L KE per bioreactor.

2.4. Measurement of malondialdehyde (MDA) and prolin content

Measurement of the malondialdehyde (MDA) and prolin content was made using a UV-Vis spectrophotometer (Thermo BOECO S-22) at 532 nm, following Kusuma et al. (2021). Standard solutions of MDA (0 – 40 nmol) were used to determine the MDA content in the root samples, with the results being represented as nmol/0.5 g FW. The of proline absorbance was measured at 520 nm. The concentration of proline was calculated based on the standard curve produced using L-proline standard solution (0 – 300 µM), and the results represented as µM /0.5 g FW.

2.5. Measurement of the physico-chemical conditions of the culture media

Measurement was conducted during the media preparation stage and at the end of the culture period. The parameters measured included the pH, total dissolved sugar, and conductivity of the media. The pH of the media was measured using pH indicator paper on a 4-7 scale (Merck), while total dissolved sugar was measured with a hand refractometer Brix scale 0-10 (Atago Master 10 T), with the results represented in percentage terms. The conductivity of the media was measured using a hand conductivity meter (Ezodo Cond521), and the results represented as µS/cm.

2.6. Analysis data

Data were analyzed based on (Kusuma et al. 2021) using descriptive quantitative and one-way analysis of variance ($p < 0.05$), followed by Duncan's multiple range test (DMRT, $p < 0.05$). The statistical analysis was performed using IBM-SPSS version 21 software.

3. Results and Discussion

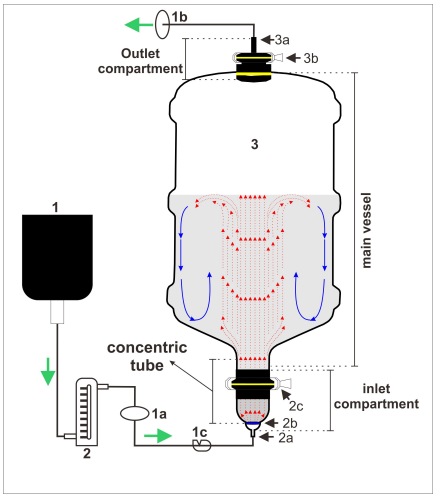
3.1. Bioreactor development

In this study, a prototype pilot 19 L capacity bioreactor for adventitious root production has been successfully developed using the working principle of the airlift bioreactor system (Figure 1b). An airlift bioreactor is one with a working system which utilizes the movement of air that enters the reactor from an inlet port at its bottom, which then passes through a liquid fluid (liquid medium) as bubbles and exits through an outlet port in the upper side (Singh et al. 2014).

The bioreactor was modified with the adjustment of the working system based on the BTBB. The pilot bioreactor design also has a concentric tube located at the bottom of the main body, in line with the BTBB design of (Paek et al. 2001). The galon is positioned upside down, with its mouth installed with concentric tube compartments for the air inflow. In addition, the upper part of the galon was modified by installing compartments for air outflow.

The 19 L pilot bioreactor was constructed by combining several compartments, namely (1) an air inlet consisting of concentric tube was made from borosilicate glass (6 cm in diameter and 10 cm in height), a glass sparger (3 cm in diameter and 60 µm in pore size), and both installed in a stainless ring; (2) a reactor body was developed from container called gallon; and (3) an air outlet compartment consisting of stainless connectors (5 cm in diameter). The schematic of the original work system and construction is presented in Figure 1a.

The bioreactor has two main areas based on the airlift bioreactor system, namely the riser and downcomer areas.



(a)

(b)

FIGURE 1 Schematic of the working system and construction of the 19 L pilot bioreactor. (a) Schematic of the installation and working system (description : 1 = Air pump; 2 = airflow meter; 3 = bioreactor body; 1a – 1b = air filter; 1c = hose clamp; 2a = inlet port; 2b = sparger; 2c – 3b = compartment clamp; 3a = outlet port; red arrow = riser area; blue arrow = downcomer area; green arrow = airflow direction); and (b) Image of the 19 L pilot bioreactor installation.

The riser area is where the liquid is propelled by the pressure of the bubbles so that there is an upward movement in the liquid medium. On the other hand, the downcomer area is where the liquid moves inward (to the bottom of the reactor) due to the pressure of the air when the bubbles disappear on the surface of the liquid medium.

The bioreactor has a simple design and working system. When the air pressure from the pump enters through the sparger pore, the air will turn into tiny air bubbles. The air pressure helps agitation, and at the same time provides oxygen to the medium. The bubble agitation in the medium applies low shear stress on the roots because there is no mechanical stirring. A large capacity bioreactor provides enough space for roots to grow optimally compared to one with a small capacity. Based on the working system, the pilot bioreactor has been successfully developed with the same working system as the previous bioreactor described developed by Paek et al. (2001).

Paek et al. (2001) detail the advantages of airlift bioreactors for adventitious root culture, including (a) low shear stress; (b) simple construction and design; (c) production of a long-term culture with low risk of contamination, as it does not require a mechanical stirrer; and (d) low energy requirements. The circulation system and the hydrodynamic movement of the bioreactor are easy to control because the circulation and agitation are determined by the air or gas flow rate, which can be controlled by an airflow meter.

3.2. Bioreactor sterilization and durability test

To remove contaminants, sterilization using an autoclave system was conducted. In this processes, the steam pressure must be controlled to achieve sterile conditions. This pressure during sterilization also impacts the durability of a bioreactor made of polycarbonate plastic. Therefore, durability tests are performed to ensure the bioreactor is not damaged by the steam pressure. The optimization bioreactor sterilization and bioreactor condition is detailed in Table 1.

A hot steam pressure of 0.18 MPa is suitable for sterilizing bioreactors and culture media. After sterilization, we confirmed that contamination was not found in the culture media for 20 days. In addition, there was no color change in the media during the observation period. Changes in the physical-chemical conditions of culture media are observed at harvest time. The pH of the media ranged between 5 - 5.5, with a conductivity of 3200-3400 µS/cm, and dissolved sugar of 2.6 - 2.8% (Figure 6). These results showed the same pattern as that in the previous study of Kusuma et al. (2021).

Pressure lower than 0.18 MPa for 60 min was not effective for the sterilization of the bioreactor since the medium was contaminated with bacteria at 3 to 15 day intervals after sterilization. In contrast, the sterilization methods decribed in previous reports were performed at 121 °C (approximately 0.115 MPa) for 15 min (Kusuma et al. 2021) or for 20 min (Saiman et al. 2012) to achieve sterile conditions. The sterilization steam pressure in this study was not effective in obtaining sterile conditions in the culture media, although the sterilization duration was longer. Therefore, the results obtained are different from previous reports. On the other hand, a pressure higher than 0.18 MPa could damage the bioreactor vessel.

3.3. Evaluation of bioreactor performance in the adventitious root culture of G. procumbens

3.3.1 Effect of different chemical grades on adventitious biomass

The results of the root morphology from the bioreactor are presented in Figure 2. The optimal root biomass was achieved using MS-Tek media, with yields of 332.50 g FW and 26.36 g DW. The biomass yields were lower in the MS-PA medium, in which the biomass yields were 225.00 g FW and 19.38 g DW (Figure 3).

The proliferation of adventitious roots in the MS-Tek medium had a faster growth rate than that of those in the MS-PA media. The root growth ratio in each treatment can be associated with the results of the root morphology, as shown in Figures 2a-b. The root growth ratio in the MS-Tek and MS-PA media resulted in 12.65-fold and 9.04-fold increases respectively, compared to the initial inoculum (Figure 3).

3.3.2 Bioactive adventitious root compound productivity

The results show that the root culture scale-up using the 19 L bioreactor improved the productivity of the bioactive compounds. Both medium treatments displayed different bioactive compound productivity, with the production of phenolics and flavonoids being higher in MS-PA than in

TABLE 1 Evaluation of sterility and the media contamination time of the 19 L pilot bioreactor for 20 days after sterilization.

Pressure	Temperature	Time	Media sterility		d	Ct	Bc
(MPa)	(°C)	(min)	Sterile	Contamination			
0.12	123	60	No	Yes	3 – 7	Bacteria	N
0.15	127	60	No	Yes	10 – 15	Bacteria	N
0.18	131	60	Yes	No	-	-	N
0.20	133	60	ND	ND	ND	ND	D

N = Normal, D = Damage in first sterilization stage
d = days of contamination, Ct = Type of contamination, Bc = Bioreactor vessel condition,
ND = non detected

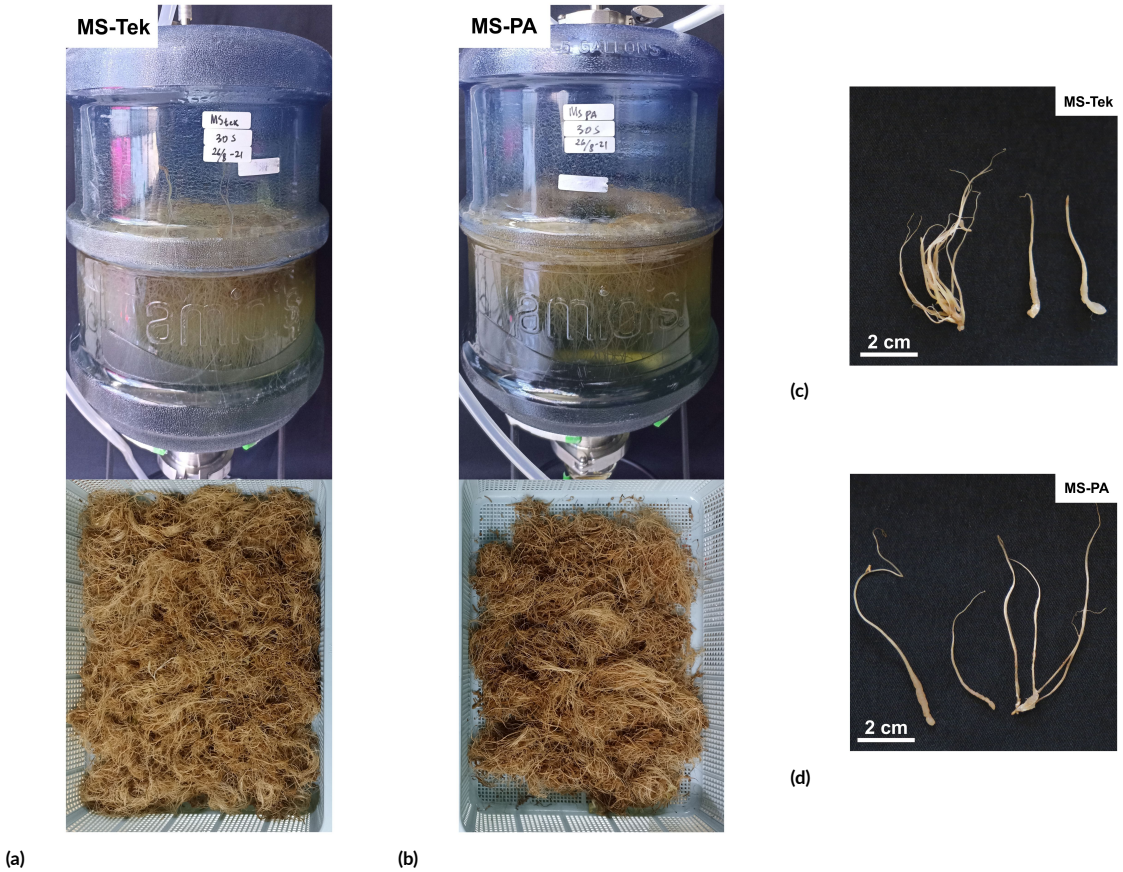


FIGURE 2 Harvest of adventitious roots of *G. procumbens* from the pilot 19 L bioreactor: (a) from the MS-Tek medium; (b) from the MS-PA medium; (c) root morphology from MS-Tek treatment; and (d) root morphology from MS-PA treatment. Roots were harvested after the 35-day culture period.

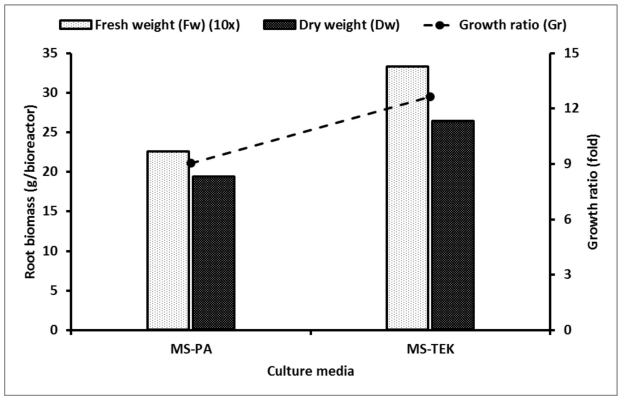


FIGURE 3 Diagram of the effect of MS macronutrient chemical grade variation on adventitious root biomass over a 35-day culture period. MS-PA = MS macronutrient using analytical-grade chemical; MS-Tek = MS macronutrient using technical-grade chemical.

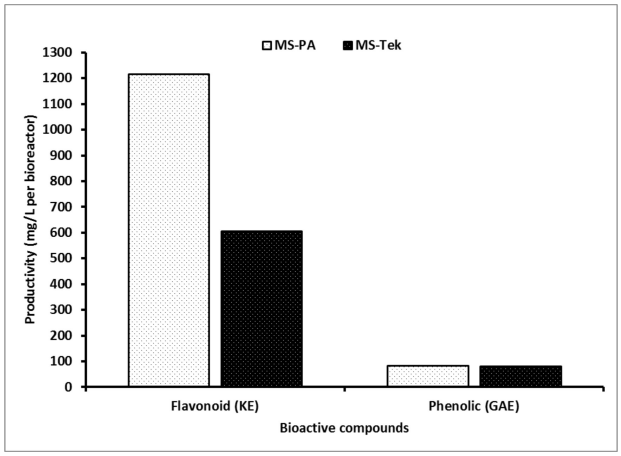


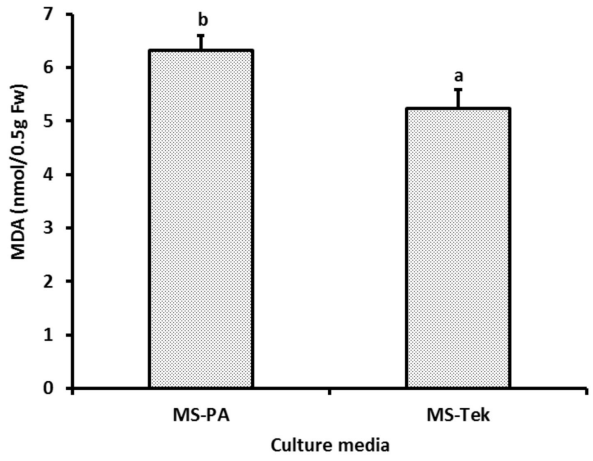
FIGURE 4 Diagram of bioactive compound productivity from adventitious root culture in the pilot 19 L bioreactor using varied MS macronutrient chemical grades after the 35-day culture period. MS-PA = MS macronutrient using analytical-grade chemical; MS-Tek = MS macronutrient using technical-grade chemical.

MS-Tek (Figure 4).

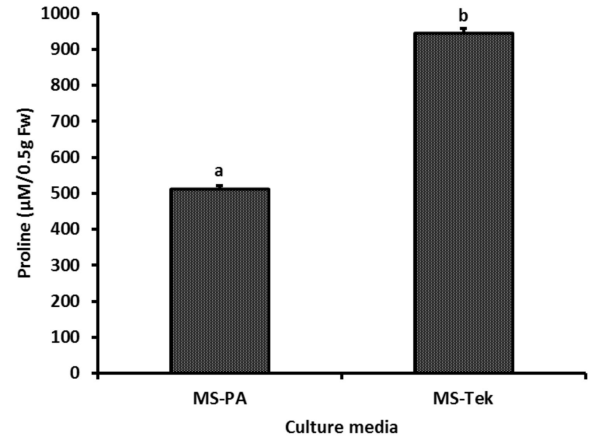
The productivity of phenolics in roots with MS-PA and MS-Tek treatments was 82.25 mg/L and 81.64 mg/L GAE per bioreactor respectively, indicating that MS-PA was only slightly higher than MS-Tek. However, the productivity of flavonoids in roots with MS-PA treatment (1216.63 mg/L KE) was twice as high as that in MS-Tek (606.88 mg/L KE). The productivity of bioactive compounds was strongly influenced by biomass yield. Therefore, a high biomass yield of scaled-up root culture is also important in increasing the level of bioactive compounds.

3.3.3 Measurement of adventitious root MDA and proline

Measurement levels of MDA and proline were used to estimate physiological root conditions during culture using the pilot bioreactor. The measurements were significantly different ($p < 0.05$), with both sets showing opposite results in each treatment (Figure 5). The MDA levels of roots in the MS-PA treatment (6.31 nmol/0.5 g FW) were higher than those in the MS-Tek treatment (5.23 nmol/0.5 g FW) (Figure 5a). In contrast, higher proline levels were detected in the MS-Tek treatment (943.01 µM/0.5 g FW) than in the MS-PA treatment (511.71 µM/0.5 g FW) (Figure 5b). The different patterns in the physiological root conditions are believed to have been affected by several environmental factors in the root culture, such as the chemical grades of the medium composition and aeration.



(a)



(b)

FIGURE 5 Diagram of physiological change: (a) MDA levels; (b) proline levels in adventitious roots from a pilot bioreactor culture using varied MS macronutrient chemical grades in a 35-day culture period. MS-PA = MS macronutrient using analytical-grade chemical. MS-Tek = MS macronutrient using technical-grade chemical. Bar = stardard deviation. a,b = the graph followed by the different letters is significantly different based on DMRT based on DMRT ($p < 0.05$).

3.3.4 Changes in the physico-chemical conditions of the culture media

The pH of the root culture in each different treatment medium changed, but was relatively stable in the range of 5 – 5.5 after the 35-day culture period (Figure 6a). The conductivity of the medium during preparation had different values for each treatment. The conductivity of the MS-PA medium (4648 µS/cm) was higher than that of the MS-Tek medium (4461 µS/cm), but both treatments shoiwed the same pattern (Figure 6b). The total dissolved sugar concentration in the MS-PA and MS-Tek shiwed different changes in the culture media. The total dissolved sugar concentration in the MS-Tek medium was lower (2.6 percent) than that in the MS-PA medium (2.8 percent) (figure 6c). Changes in the physico-chemical conditions of the culture medium are considered to be related to the root biomass from the culture using the pilot bioreactor.

3.4. Discussion

3.4.1 Bioreactor development for adventitious root production

In this study, the pilot bioreactor model made from galon had a cylindrical shape with an airlift working system. In a previous study, Paek et al. (2001) modified airlift bioreactor installed with a concentric tube and a sparger at the bottom of the vessel reactor had the advantage of reducing shear stress. However, Murthy et al. (2014) explained that there was an effect of the design of the bioreactor model on the initial oxygen transfer coefficient in the bioreactor, which could affect the growth of propagules in the bioreactor. A bulb-type bioreactor with a spherical (balloon) bioreactor construction and a long concentric tube at the bottom of the reactor vessel was found to be a suitable model for the adventitious root culture of *P. ginseng* (Kim, Yun-Soo and Hahn, Eun-Joo and Paek 2004).

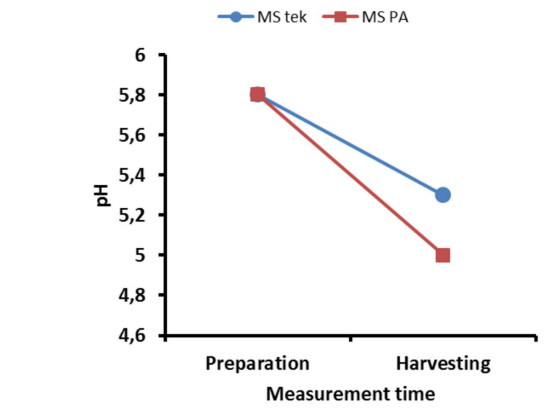
In line with bioreactor development, balloon or bulb-type airlift bioreactors have been developed with different large capacity models; for example, a large diameter cylinder bioreactor with a 1000L capacity. This has a concentric tube at the bottom of the vessel, which is a feature of the ballon-type bioreactor. It has a different appearance to the initial prototype (balloon form). However, the working system and features of the prototype were adapted for cultures at larger capacities (Murthy et al. 2014).

Lee (2009) described a suitable choice for an airlift bioreactor model for small-scale adventitious root culture production, with the model resembling a galon, so known as galon-like bioreactor. Lee (2009) found that the production of *P. ginseng* root culture at the laboratory scale using a 20L galon-like bioreactor model was reliable. Park et al. (2013) and Ghimire et al. (2017) also reported the use of a galon-like bioreactor to optimize ginseng root culture. In a recent study, Rahmat et al. (2021) refer to the term 'galon-like bioreactor' in relation to a 20L balloon-type bubble bioreactor (BTBB), which was successfully used to scale up the root culture of *R. glutinosa*.

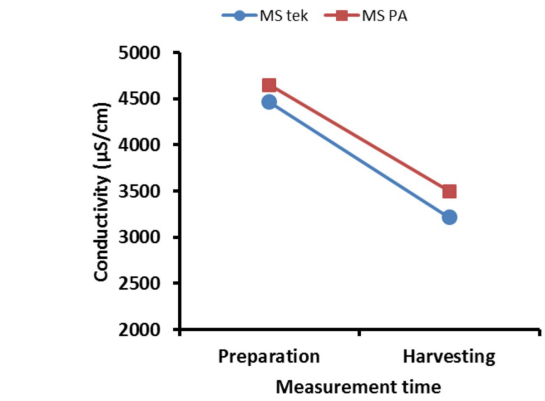
The productivity of adventitious root culture using

large-scale bioreactors has varied. It has been indicated that each plant species has different productivity optimization. In comparison, the dry weight of the roots of *G. procumbens* obtained from the 19 L pilot bioreactor was higher than that of *R. glutinosa* root culture, based on the study by Rahmat et al. (2021). Therefore, these results can be used as a reference to show that the bioreactor developed in this study is feasible for use as a scale-up tool for *G. procumbens* adventitious root culture.

Therefore, the capacity of the bioreactor is sufficient for use as a tool to produce biomass and bioactive compounds from adventitious roots of *G. procumbens* at the laboratory scale. The production process at such a scale can be arranged to use several bioreactors with a capacity of 19 L. This has advantages in terms of simple technology, low investment costs, and simplification of the handling production, thus reducing contamination levels.



(a)



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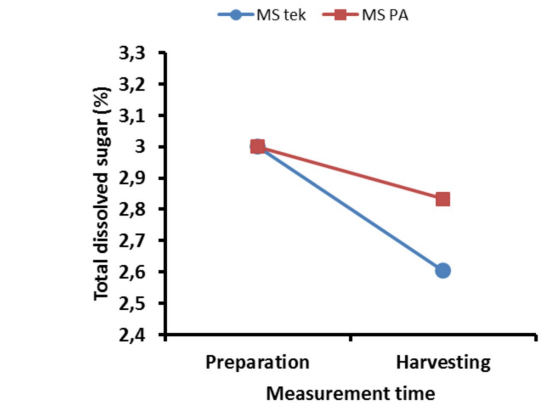

(c)

FIGURE 6 Graphics physico-chemical of culture media: (a) pH; (b) conductivity; (c) total dissolved sugar change in the MS culture medium with chemical grade variation. MS-PA = MS macronutrient using analytical-grade chemical; MS-Tek = MS macronutrient using technical-grade chemical.

3.4.2 Bioreactor sterilization and durability test

Based on the observations, sterilization at 0.18 MPa did not have a negative effect on the construction of the bioreactor or the culture media. The bioreactor suffered no damage or change from during the sterilization process nor was there any drastic effect on the physical and chemical conditions of the culture media. Physically, there was no change in the culture media color which indicated damage to the medium, such as browning due to caramelization by high temperatures. Since the media were exposed to heat during sterilization, the pH decreased, while conductivity and dissolved sugar increased (Kusuma et al. 2017, 2021). However, physical-chemical observations after sterilization were not made in this study. Nevertheless, at harvest time (Figure 6a) it was shown that the pH of the culture medium was stable, in the range of 5 - 5.5, in line with the previous study of Kusuma et al. (2017). On the other hand, the decrease in conductivity and dissolved sugar indicated nutrient uptake by the root (Kusuma et al. 2021).

However, sterilization treatment using a pressure of 0.20 MPa resulted in damage to the bioreactor, meaning the reactor vessel was not reusable. The polycarbonate plastic construction of the bioreactor has a limited tolerance to heat during sterilization. Based on the explanation of Sastri (2022), PCs plastic material can be sterilized using an autoclave with a temperature range of 115 °C – 135 °C at different times. PCs plastic will reach the heat distortion temperature (HDT) point at 135 °C – 140 °C, which causes polymer changes due to load. Based on the pressure gauge on the autoclave, a pressure of 0.20 MPa is equivalent to a temperature of 133 °C (Table 1). It is believed that the reactor vessel reached the HDT point at a temperature of 133 °C, so the vessel deformed and could not return to its original shape.

3.4.3 Effect of different chemical grades on adventitious root growth

The chemical reagents used in MS media for research commonly involve analytical grade materials, apart from sucrose and agar, as these can be obtained easily (Prakash et al. 2004). We found some chemical reagents for macronutrients (NH_4NO_3, KNO_3, $MgSO_4$, $CaCl_2$, and KH_2PO_4) at low prices and these were obtained easily in

the market. We obtained two different brands of these cheap chemicals. We did not find an official certificate of analysis (CoA) of the chemical products, only the chemical specifications on the product packaging label (Supplementary Appendix 1). On the other hand, expensive chemicals are grouped as analytical-grade chemicals, with official CoAs available from the manufacturers' websites according to the product batch number (Supplementary Appendix 2).

Based on the reasons above, we attempted to compare the MS medium macronutrient reagent grades for the adventitious root cultures. This applied only to the macronutrient reagents, as these were needed in large quantities for the MS media. This study applies technical-grade material to the macronutrient composition in the MS media for adventitious root culture production. We found that technical-grade chemicals have the potential as alternative materials for root biomass production.

Morphological observation showed that root growth in the bioreactor had a similar pattern to that described by Kusuma et al. (2021). The root growth in the bioreactor had characteristic overlaps and twists in the form of the root rolls (Figure 2a-b). The root morphology in each treatment had normal characteristics, with slightly different branching and root lengths. The MS-Tek medium treatment produced more root branching with short and thick characteristics (Figure 2c), while the MS-PA treatment resulted in less root branching with long and thin characteristics (Figure 2d).

Figure 4 shows that technical-grade chemicals can provide sufficient nutrients for root growth, enabling them to have high biomass. The presence of macronutrients in the culture medium has a great influence on root growth. Murthy et al. (2014) found that the concentration of macronutrients in the medium greatly affected the biomass of ginseng roots. In this study, the concentration of nutrient ions in the culture medium differed between the MS-Tek and MS-PA media (Figure 6b). MS-Tek had a lower nutrient ion concentration than MS-PA. The difference in nutrient ion concentration may be caused by variations in the reagent constituents which affect the purity level of the two types of chemical. The composition of reagents is suspected to lead to different responses in root cell affinity to nutrient ions. This affinity may be related to the activity of enzymes which are responsible for transforming nitrogen ions into amino acids for growth (Lulu et al. 2015). Therefore, there were differences in root biomass yield in each treatment.

Non-analytical grade chemicals may not be suitable for basic research or analysis in tissue culture. However, they can be used as an alternative to chemicals for culture media in plant tissue culture production; for example, producing *G. procumbens* adventitious root biomass. In general, technical grade chemicals have been widely used in applied and commercial research in tissue culture activities. Several studies have found that non-analytical chemicals, such as commercial chemical fertilizers, can be used as alternative compounds for media culture for orchid production (Hasanah et al. 2014; An et al. 2021). Another study also found that non-analytical chemicals could be formulated as an alternative medium to reduce production costs by up to 73.20 percent in banana culture production (Dhanalakshmi and Stephan 2016). However, no studies have specifically discussed the effect of technical-grade chemicals used in plant tissue culture research. Therefore, further research should examine the effects of such chemicals in culture media.

3.4.4 Effect of different chemical grades on bioactive compound productivity

Different chemical grades are factors that are believed to influence the different yields of bioactive compounds in the adventitious roots of *G. procumbens*. The difference between analytical grade chemicals and technical grade ones is the presence of chemical impurities in technical-grade chemicals that could reduce the purity level of the chemical (Prakash et al. 2004).

The decrease in bioactive compound productivity may be because chemical impurities affect the biosynthesis of bioactive compounds in the root. The other possibility is that there are differences in the sensitivity of root cells and their metabolism to nutrient ions in different chemical grades, so roots have different responses in secondary metabolite biosynthesis. The affinity of root cells to nitrogen ions is suggested to occur more responsively in MS-Tek compared to MS-PA media, so roots reach maximum growth. Therefore, the flavonoid productivity in MS-Tek media is lower than in MS-PA (Figure 4).

A high affinity to nitrogen ions has been considered to affect flavonoids biosynthesis in plant organs. Deng et al. (2019) explain that the accumulation of carbohydrates and flavonoids is mediated by nitrogen levels, which indicates that nitrogen can affect the balance of the C:N ratio and flavonoids accumulation in plants. Therefore, this balance can control not only the carbon portion in the amino acid and carbohydrate biosynthetic pathway, but also in the flavonoids biosynthetic pathway. However, the effect of different chemical grades on the biosynthesis of adventitious root metabolism, both for primary and secondary metabolisms, is not yet fully known.

In addition, the low levels of bioactive compounds in the M-Tek medium treatment suggests that root growth might not be optimal. The root metabolism focuses on primary metabolism rather than producing secondary metabolites. This assumption is in line with the results from malondialdehyde (MDA), which were lower than those of MS-Tek. The lower MDA level (Figure 5a) indicates no stresses in the root growth, enabling the roots to achieve optimal growth, with no reason to produce secondary metabolites at high levels. Moreover, based on the character of the adventitious root growth, the MS-Tek medium provides nutrients for optimal root growth.

On the other hand, higher bioactive compounds were detected in the MS-PA medium. In line with the results of the MDA measurement (Figure 5a), there was an as-

sumption that a high MDA level meant that stress had occurred in the roots. One of the root responses to stress is the production of antioxidant compounds (Baque et al. 2014). Antioxidant activity is usually associated with secondary metabolites that have antioxidant potential, such as phenolic compounds (Samuoliene et al. 2017). Therefore, high levels of total phenolics and flavonoids were measured in roots from cultures using the MS-PA medium (Figure 4). Similar results were obtained by Baque et al. (2013), who found that the increase in phenolic and flavonoid compounds was strongly influenced by the MDA levels measured in the roots of *M. citrifolia*.

3.4.5 Effect of different chemical grades on levels of MDA and proline

MDA and proline levels in the adventitious roots G. procumbens were significantly different (Figure 5). We suggest that the pattern of root growth density in the bioreactor was an indirect factor affecting MDA levels in the roots as root density is related to root growth, which in turn is influenced by each culture medium using different grade chemicals. Root mass density in the MS-Tek medium treatment naturally caused damage to root cells, together with a slight effect of shear stress in the bioreactor. Shear stress from aeration contibutes to the emergence of reactive oxygen species (ROS), which cause cell membrane damage, and can be determined through MDA levels (Baque et al. 2013). The MDA levels from adventitious roots in MS-Tek are in line our previous studies, with low MDA levels also detected in the optimal adventitious root growth of *G. procumbens* (Kusuma et al. 2021).

On the other hand, the MDA content of roots from the MS-PA medium treatment was higher, presumably due to the high accumulation of H_2O_2. High shear stress could emerge in low root density due to aeration. It is suggested that higher accumulation of ROS in the roots results in the accumulation of H_2O_2 and more damage to the root cell membrane. This phenomenon is in accordance with research by Baque et al. (2013), who found that low root density resulted in high MDA levels, which were associated with a high accumulation of H_2O_2 due to aeration. It can be assumed that this high accumulation of H_2O_2, promoted by low root density conditions, can trigger high MDA levels in the roots.

Free proline is always detected in adventitious root cultures because proline functions as an osmoregulator at the cellular level when the plant is under stress due to submersion or under normal conditions (Kishor et al. 2015). The natural response of the roots when adapting to the environment is the accumulation of proline in the root tissue. Proline levels were higher in the MS-Tek treatment compared to the MS-PA. The use of technical grade chemicals may be a factor in high levels of proline. Impure chemicals from technical grade material in the MS-Tek medium may contribute to increased proline levels as such high levels are identical to the mechanism of root response to excessive mineral concentration stress (osmotic stress) in liquid media (Baque et al. 2013).

4. Conclusions

It is concluded that the pilot bioreactor has been successfully developed and can be used to produce adventitious root *G. procumbens* through *in vitro* culture. The results of the root culture experiments using different grades of reagent show that technical grade chemicals (MS-Tek medium) are suitable for increasing root biomass production. On the other hand, analytical grade chemicals (MS-PA medium) are suitable for improving phenolic and flavonoid compounds. However, further study is necessary to evaluate the root growth kinetics and nutrient (technical grade chemicals) ion uptake to obtain suitable media formulation and culture conditions for the production of metabolite compounds from adventitious roots at an affordable cost.

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Authors’ contributions

DYK designed the study, carried out the laboratory work, data analyzed, wrote the manuscript. ANK and YSWM supervised the research, reviewed the manuscript. YSWM acquired the funding for the research. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no conflict of interest.

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