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Research Article
Biomass and Flavonoid Production of *Gynura procumbens* Adventitious Roots Influenced by MS Salt Strength and Nitrogen Source in a Balloon-type Bubble Bioreactor

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Abstract

Background and Objective: *In vitro* culture of *Gynura procumbens* adventitious roots has been carried out by various methods, sucrose concentrations and elicitation. However, adventitious root yields and flavonoid content have not yet been maximized. This study was aimed of this study was to enhance of biomass and flavonoid production from *G. procumbens* adventitious roots influenced by salt and nitrogen supply in Balloon Type Bubble Bioreactor (BTBB). **Materials and Methods:** Adventitious-root explants were obtained from leaves and were grown in MS medium supplemented with 5 mg L^{-1} of IBA. Then, 2 g of adventitious roots were cultured in a BTBB containing MS medium of one of four different salt strengths (½×, ¾×, 1× and 2× MS) or one of six ammonium:nitrate ratio treatments (0:30, 10:20, 15:15, 20:10, 30:0 and normal MS medium). **Results:** The highest adventitious root biomass yield was obtained in ½× strength MS medium and an ammonium nitrate ratio of 10:20, whereas the highest flavonoid content was obtained in 2× strength MS and an ammonium nitrate ratio of 0:30. Adventitious root biomass showed a maximum 18-fold increase compared to the initial inoculum and flavonoid production showed a 1.6-fold increase compared to the roots of the mother plant. **Conclusion:** These results can be employed as a basis for developing large-scale cultures because they provide information on the economical use of media and reduced application of ammonium nitrate.

Key words: Balloon-type bubble bioreactor, *Gynura procumbens*, salt strength, adventitious roots, ammonium nitrate ratio, biomass, flavonoid

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The demand for plants with high-value bioactive compounds for pharmaceutical use, healthy food and cosmetic products continue to increase together with the recognition of their pharmacological benefits[1]. Along with the growing use of plant bioactive compounds, the availability of plants in their natural habitats has decreased year on year due to the increasing world population and environmental pollution. The amount and quality of bioactive compounds obtained from plants grown under conventional cultivation methods are limited because their availability is influenced by harvest time and environmental conditions[2,3].

The pharmaceutical industry cannot rely on conventional cultivation methods for continuous supplies of raw medicinal plant resources. Therefore, alternative methods for producing medicinal plants are needed to meet the demand and for large-scale propagation of plant cells and organs of uniform quality[4]. *In vitro* culture techniques are the most effective methods for producing large amounts of biomass and secondary metabolites. Plant organs, including adventitious roots, hairy roots and embryos, have been widely cultured. Among these culture techniques, adventitious root cultural has a high proliferation rate and has great potential for the production and accumulation of useful secondary metabolites[3,5]. Commercial production of secondary metabolites using large-scale bioreactors is more efficient than conventional culture. Balloon-Type Bubble Bioreactors (BTBBs) have good aeration and agitation and are designed to prevent foaming and cell growth on the bioreactor wall[6]. The BTBB is the most effective bioreactor for adventitious root culture compared to mechanically agitated bioreactors[7].

Gynura procumbens (Lour.) Merr. (Asteraceae) is a medicinal plant whose leaves are also consumed as a vegetable. The plant is commonly found in the tropical areas of China Indonesia, Thailand, Malaysia and Vietnam[8]. *G. procumbens* extract has been reported to have various pharmacological activities, such as antioxidant[9-12], anticancer[13-15] and anti-inflammatory activity[16,17]. Efforts to increase biomass and flavonoid production in adventitious roots of *G. procumbens* in BTBB have been carried out with various treatments, such as the addition of sucrose at different concentrations[18] and elicitation[19]. The results of these treatments showed that adventitious root biomass increased 10-12-fold compared to the initial inoculum.

Optimization of the *in vitro* culture medium is necessary to meet the nutritional needs of explants for rapid growth and enhanced production of secondary metabolites. Interactions between nutrients in low-salt-strength culture enhanced the availability of ions to the roots, so that adventitious root cultures of *Echinacea angustifolia* in quarter- and half-strength Murashige and Skoog (MS) medium showed increased accumulation of biomass and secondary metabolites[5,20]. In another study, half- and double-strength MS medium produced high levels of methyl eugenol in the shoot culture of *Ocimum basilicum*[21], while full-strength MS medium produced the highest amount of biomass and withanolide A from cell suspension culture of *Withania somnifera*[22]. *Eleutherococcus koreanum* adventitious root culture in half-strength MS produced increased biomass and eleutheroside B and E[23], while adventitious root culture of *Morinda citrifolia* also produced similar results for biomass and bioactive compounds[24]. Therefore, the appropriate concentration of culture medium constituents is crucial for the growth of isolated plant cells and organs.

Another strategy that can be used to optimise adventitious root culture is nutrient manipulation. Nitrate, present in tissue culture media, is a source of the macronutrient nitrogen. Nitrates play a role in suppressing key enzymes in the starch biosynthetic pathway as well as in the assimilation of nitrogen into carbon skeletons[25]. Nitrate increases nitrogen assimilation, resulting in a corresponding increase in the amount of protein and enhancement of plant growth and secondary metabolite content. The nitrate added to tissue culture medium can be in the form of potassium nitrate and ammonium nitrate[26]. MS medium, which is widely used for *in vitro* culture, contains a higher amount of nitrogen (60 mM) compared to other *in vitro* culture media. The total nitrogen source and NH_4^+:NO_3^- ratio affect biomass and bioactive compound production, for example in cell culture of *Panax quinquefolius*[27], adventitious root culture of *P. Ginseng*[28], cell suspension, adventitious root and hairy root culture of *W. Somnifera*[29-31] and adventitious root culture of *Glycyrrhiza uralensis*[32]. This study was aimed to enhance biomass and flavonoid production of *G. procumbens* adventitious roots in BTBBs under different MS salt strengths and nitrogen sources.

MATERIALS AND METHODS

The study was conducted in Plant Tissue Culture, Biology Department, Faculty of Science and Technology, Universitas Airlangga, Surabaya, East Java, Indonesia, from January to October 2019.

Adventitious root induction: *G. procumbens* plants were obtained from the collection of the Indonesian Institute of Sciences, Purwodadi Botanical Garden, Pasuruan, East Java,

Indonesia, with identification number 1610/IPH.06/HM/XI/2015. Adventitious root induction was done according to Manuhara *et al.*[18] The leaves of *G. procumbens* were cleaned with detergent for 5 min and then rinsed thoroughly with tap water. Before preparing explants, it was necessary to sterilise the leaves by soaking them in Clorox 10% (v/v) for 10 min before finally rinsing three times with sterilised distilled water. The explants were cut into their final size of 1 cm^2, then were planted in the MS solid medium was supplemented with 5 mg L^{-1} of IBA, 30 g L^{-1} of sucrose and 7 g L^{-1} of agar. The cultures were maintained under low light at a temperature of 25°C. The adventitious roots produced by the leaf explants were then collected after 21 days for use as explants in the BTBBs.

Adventitious root culture in balloon-type bubble bioreactors: BTBBs with 1 L capacity were used in this study; 600 mL MS liquid medium was added to each BTBB for inoculation of 2 g of adventitious roots. The MS liquid medium was previously supplemented with 5 mg L^{-1} of IBA and 30 g L^{-1} of sucrose. For 28 days, the cultures were incubated in low light and low ambient temperature, with an aeration rate of 0.2 vvm. The pH of the medium was assessed weekly. This protocol was done according to Manuhara *et al.*[18] There were two experimental factors in this study: salt strength and ammonium to nitrate ratio of the MS medium. MS salt strength involved four treatments of half, three-quarter, full and double strength (½×, ¾×, 1× and 2×). The different treatments for ammonium:nitrate ratio were 0:30, 10:20, 15:15, 20:10, 30:0 and normal MS medium (21:19 control).

Extraction and analysis of flavonoids: The adventitious roots produced in each treatment were oven-dried at 50°C for 3 days and ground with mortar and pestle; 0.5 g of the dried and powdered biomass was immersed in 10 mL of methanol. This procedure was repeated twice for each treatment. The extracts were filtered before being finally concentrated to 6 mL at room temperature. In order to expunge any non-polar compounds, the methanol extracts were partitioned with 1:1 n-hexane. This was followed by partitioning with 1:1 ethyl acetate. Finally, ethyl acetate extracts were used to analyse flavonoid content using a spectrophotometer (BOECO S-22, Germany). The compounds used in this study as reference standards were kaempferol (Sigma, USA) and quercetin (Sigma, USA). The total flavonoid content was assessed by UV colorimetry[10]. About 900 µL of each ethanol extract was mixed with 10 µL of distilled water to make 1 mL of ethanol extract. Next, a 0.25 mL sample of each treatment was mixed with 1.25 mL of distilled water and 75 µL of $NaNO_2$ solution. This extract was left for 6 min before finally being added to 0.15 mL of a 10% $AlCl_3$ solution and then incubated for 5 min. In addition, to make the volume up to 25 mL, 0.5 mL of 1 M NaOH and distilled water were added. The absorbance of the mixed solution was measured at 510 nm using a UV-Vis spectrophotometer (BOECO S-22, Germany). This quantification also used kaempferol and quercetin as standard compounds.

RESULTS

Effect of MS salt concentration on biomass and flavonoid production: Table 1 shows the effects of different salt strengths of MS medium on biomass and flavonoid production in the adventitious roots of *G. procumbens*. Biomass production was higher at ½×, ¾× and 1× MS salt strengths than at high salt strength (2×MS), the adventitious root biomass was 25.50 g FW, 23.42 g FW, 22.72 g FW, respectively (Table 1). The highest fresh and dry weights were achieved at ½×MS after being cultured for four weeks; fresh weight was 18 fold compared to the initial inoculums. Adventitious root cultures at high salt strength (2×MS) produced very little extra biomass; fresh weight was 2.44-fold compared to the initial inoculums. Salt strength of medium also influenced the production of flavonoids (quercetin and kaempferol). The highest flavonoid content was in high strength treatment (2×MS). This treatment had higher flavonoid content than the mother plant. The lowest flavonoid content was found in the 1×MS treatment. The growth and morphology of *G. procumbens* adventitious roots cultured in BTBBs for 28 days in media of different salt strength are shown in Fig. 1a-d.

Table 1: Effects of the salt strength of the MS medium on biomass and flavonoid production in adventitious roots of *Gynura procumbens* after 28 days of culture in BTBB

MS salt strength	Initial inoculum (g FW)	Fresh weight (g)	Dry weight (g)	Kaempferol (mg L^{-1})/g DW	Quercetin (mg L^{-1})/g DW
½×	2	25.50±6.58	1.08±0.41	29259.26	8227.78
¾×	2	23.42±1.20	0.86±0.05	11555.56	2916.67
1×	2	22.72±8.91	0.81±0.18	9074.07	2172.22
2×	2	4.88±1.23	0.48±0.02	47703.70	13761.11
Roots of mother plant				29555.6	8316.7

Fig. 1(a-d): Adventitious root culture of *Gynura procumbens* in balloon-type bubble bioreactors under different salt strengths of MS medium, (a) ½× MS, (b) ¾× MS, (c) 1× MS and (d) 2× MS, Scale bar (above) = 3 cm, Scale bar = 1 cm

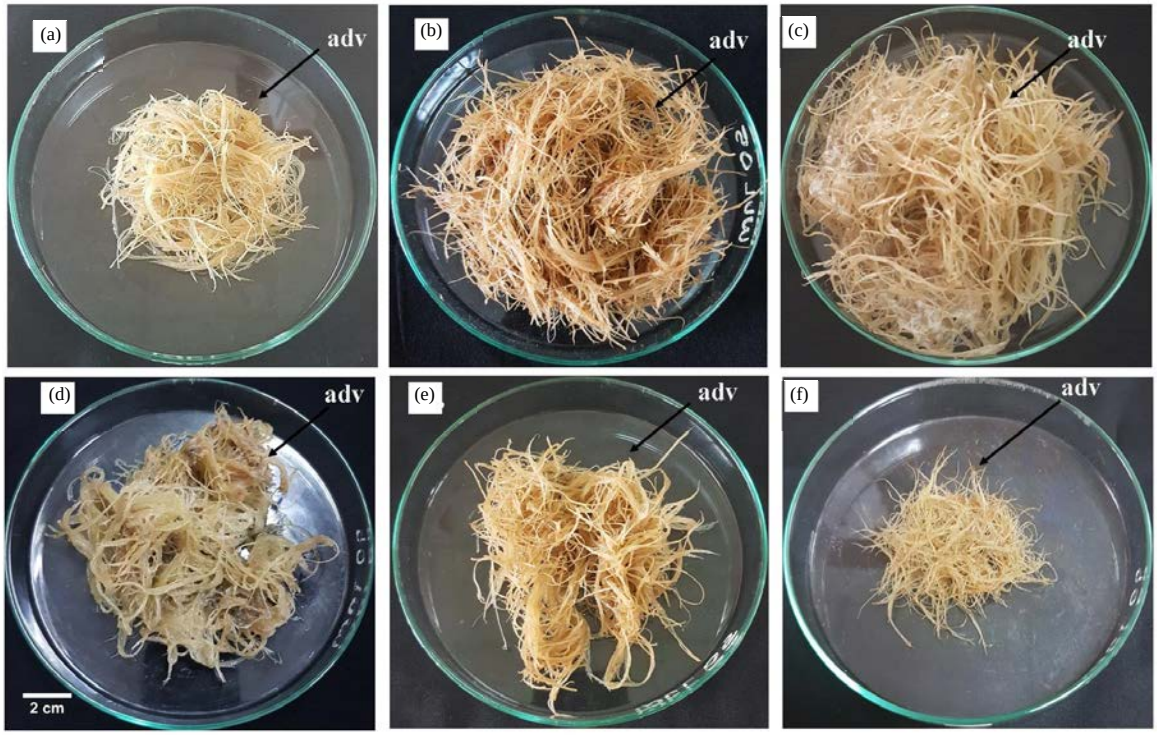

Fig. 2(a-f): Biomass of *Gynura procumbens* adventitious roots after 28 days of culture in MS medium with different ratios of ammonium:nitrate, (a) 21:19 (control), (b) 0:30, (c) 10:20, (d) 15:15, (e) 20:10 and (f) 30:0, adv: Adventitious roots

Table 2: Effects of nitrogen source on biomass and flavonoid production in adventitious roots of *Gynura procumbens* after 28 days of culture in balloon-type bubble bioreactors

NH_4^+:NO_3^- ratio	Initial inoculum (g FW)	Fresh weight (g)	Dry weight (g)	Kaempferol (mg L^{-1})/g DW	Quercetin (mg L^{-1})/g DW
21:19 (control)	2	23.24±0.18	1.42±0.38	192.00	1006.66
0:30	2	31.05±1.15	1.54±0.32	5322.00	18106.66
10:20	2	36.88±1.92	1.60±0.00	2306.00	8053.34
15:15	2	21.63±0.34	1.33±0.06	438.00	1826.66
20:10	2	20.57±0.12	1.20±0.15	387.34	1657.78
30:0	2	5.34±0.02	0.37±0.00	532.66	2142.22

Effect of nitrogen composition of MS medium on biomass and flavonoid production: The ammonium:nitrate ratio of 10:20 produced the highest biomass (36.88 g of fresh weight and 1.6 g of dry weight). This treatment increased adventitious root biomass 18.44-fold compared to the initial inoculum. The lowest biomass was found at the ammonium:nitrate ratio of 30:0 (5.34 g of fresh weight and 0.37 g of dry weight), (Table 2), the increase in biomass was only 2.67-fold compared to the initial inoculum. The morphology of *G. procumbens* adventitious roots cultured at the different ammonium:nitrate ratios for 28 days is shown in Fig. 2a-f.

The highest flavonoid (quercetin and kaempferol) content was produced by adventitious roots treated with the ammonium:nitrate ratio of 0:30, with 18106.66 mg/L/g dry weight of quercetin and 5322 mg/L/g dry weight of kaempferol. On the other hand, the lowest flavonoid content was produced by adventitious roots treated with standard MS medium (1006.66 mg/L/g dry weight of quercetin and 192 mg L/g dry weight of kaempferol).

DISCUSSION

The highest *G. procumbens* adventitious root biomass was obtained at ½× salt strength of MS medium and the lowest at 2× salt strength. In this study, ½× salt strength increased the interaction between nutrients and explants, thus increasing ion availability for root growth. This result is similar to those for adventitious root cultures of *Echinacea angustifolia*[5], *E. koreanum*[24] and *Ophiorrhiza mungos*[33]. In 2×MS medium, adventitious root growth was limited, possibly due to high osmotic pressure, which limits water absorption and mineral nutrition. Also, these conditions also lead to ion toxicity which influences cell growth[34].

At high salt strength, the residue of minerals in the medium influences water potential because it is controlled by the total mineral content, sucrose content and physical dehydration of explants[20,27,35]. The decrease in water potential as a result of physical dehydration prevents ions from being absorbed properly by roots and causes a decrease in growth and metabolism, e.g., decrease in cell elongation, biomass accumulation and biosynthesis of secondary metabolites[23,33]. In this study, the highest flavonoid content was obtained in 2×MS medium. This result is similar to that for methyl eugenol production in *O. basilicum* in ¼× and 2× salt strength of MS medium; methyl eugenol production was higher at these salt strengths than at full strength[21]. The optimum nutrient concentration of the medium is a critical determinant in controlling the growth of explants and the accumulation of secondary metabolites[7,22,36].

Nitrogen concentration affects the level of proteinaceous or amino acid products in adventitious root cultures. In this study, a low concentration of ammonium with a moderate concentration of nitrate (10:20) favored the highest accumulation of biomass production, whereas the maximum flavonoid yield was obtained in the ammonium-free medium. These results suggest that the moderate concentration of nitrate is suitable not only for optimum biomass production of adventitious roots but also for optimum accumulation of flavonoids (quercetin and kaempferol). It is a general observation that lower ammonium to nitrate ratio is more favorable for plant tissue and cell growth[37]. This is shown by studies on *Eurycoma longifolia, W. somnifera*, *Bacopa monnieri* and *Periploca sepium.* The maximum growth of *E. longifolia* adventitious roots was obtained at ammonium:nitrate ratio of 15:30[4]. Higher biomass yield of *W. somnifera* cell suspension culture was achieved when the concentration of nitrate was higher than that of ammonium[22]. In shoot culture of *B. monnieri*, the number of shoots and bacoside A content was highest in medium with low ammonium[38]. In addition, the highest *P. sepium* adventitious root biomass was obtained in medium with an ammonium:nitrate ratio of 10:20[39]. In this study, flavonoids were detected using kaempferol and quercetin as standards. This was based on the study by Kaewseejan *et al.*[10] who found four types of flavonoids, i.e. routine, myricetin, quercetin and kaempferol, in *G. procumbens* leaves from crude ethanolic extract, ethyl acetate fraction and several subfractions; kaempferol levels were higher than other types of flavonoids in the crude ethanolic extract, whereas, in the ethyl acetate fraction, the levels of these two flavonoids were almost the

same, although they were lower than the level of myricetin but higher than the levels of rutin and apigenin. In other studies, kaempferol has also been used as an indicator of the presence of flavonoids in different plant parts and the callus of *G. procumbens* and in different parts of *G. bicolor*, these results showed that the highest levels of kaempferol were obtained in the roots of *G. procumbens*[11].

In this study, the lowest biomass yield was obtained in the medium with ammonium: nitrate ratio of 30:0. The same result has been obtained for the cultures of *Glycyrrhiza uralensis* adventitious roots[32]. Ammonium as the only source of nitrogen is not advantageous for root growth, whereas nitrate as the single nitrogen source promotes better adventitious root growth. The high concentration of ammonium has an inhibitory effect on cultured cells of *P. Quinquefolium*[26] and callus growth of *Pinus strobus*[40]. This is because large amounts of ammonium in the medium are absorbed by explants and accumulate in cells. Ammonium is toxic if it is not immediately metabolized; therefore, ammonium must be maintained at low concentrations in medium[41]. Nitrate, as one of the main nitrogen sources for plants, has advantages because it is easy to move in xylem and can be stored in the vacuoles of root organs and shoots, unlike ammonium, which must be combined with other organic compounds[42]. Sources of nitrogen in the medium are obtained from potassium nitrate and ammonium nitrate. In practice the purchase of ammonium nitrate is limited. The results of this study can be used as an alternative to reducing the use of ammonium nitrate in *in vitro* culture.

CONCLUSION

The results of this study suggest that salt concentration and ammonium:nitrate ratio of MS medium effect on biomass and flavonoid (kaempferol and quercetin) production in adventitious roots of *G. procumbens* cultured in BTBBs. The highest biomass was obtained at half-strength of salt in the MS medium and ammonium:nitrate ratio of 10:20, whereas the highest flavonoid production was obtained at double-strength salt and no ammonium in the medium. These results can be employed as a basis for developing large-scale cultures because they provide information on the economical use of media and reduced application of ammonium nitrate.

SIGNIFICANCE STATEMENT

This study found that the use of MS half strength and low ammonium could increase biomass and flavonoid compounds. These results are very beneficial for the efficient use of inorganic and nitrogen sources in liquid MS medium, especially in balloon type bubble bioreactors, because these efficiencies can be used as a basis for increasing production of biomass and bioactive compounds of *G. procumbens* adventitious roots on large scale so that could save production costs.

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