Shoots Culture of Gynura procumbens (Lour.) Merr. in Balloon- Type Bubble-bioreactor Influenced by Sucrose Concentration and Inoculums Density

by Alfinda Novi Kristanti

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Research Article

Shoots Culture of *Gynura procumbens* (Lour.) Merr. in Balloon-Type Bubble-bioreactor Influenced by Sucrose Concentration and Inoculums Density

[1]Ira Nailas Saadah, [2]Alfinda Novi Kristanti, [3]Popy Hartatie Hardjo and [1]Yosephine Sri Wulan Manuhara

[1]Department of Biology, Faculty of Science and Technology, Airlangga University, Surabaya, Indonesia
[2]Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya, Indonesia
[3]Faculty of Biotechnology, Surabaya University, Surabaya, Indonesia

Abstract

Background and objectives: *Gynura procumbens* has been widely used as a traditional medicine in Indonesia. Micropropagation of this plant was important to fulfill the need of pharmaceutical industry. The aims of this study were to increasing the number of *G. procumbens* shoots in balloon-type bubble bioreactor influenced by sucrose concentration and inoculums density. **Materials and Methods:** Nodal segments of *G. procumbens* were used as explants which were grown in balloon type bubble bioreactor in MS liquid medium supplemented with 2 mg L^{-1} IAA and 4 mg L^{-1} BAP. Shoot culture were conducted under treatment of various concentration of sucrose (10, 30 and 50 g L^{-1}) and various inoculums density (5, 10 and 15 explants). **Results:** The highest multiplication of shoots and growth index were obtained at 50 g L^{-1} sucrose treatment and 5 explants as initial inoculums; there are 9.36 shoots/explants and 13.8 shoots/explants, respectively. **Conclusion:** The study found that *G. procumbens* shoots cultured in balloon-type bubble bioreactors increased shoots multiplication significantly.

Key words: Balloon-type, bubble bioreactor, *Gynura procumbens*, inoculums density, sucrose, shoot culture

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Corresponding Author: Yosephine Sri Wulan Manuhara, Department of Biology, Faculty of Science and Technology, Airlangga University, Surabaya, Indonesia Tel: 62817484872

Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Gynura procumbens is a plant that has been traditionally used as an herbal medicine and scientifically proven its extract benefits to treat various health problems such as antimicrobial[1-3], antioxidants[4-7] and anticancer[8-10]. Micropropagation of this plant was important to fulfill the need of pharmaceutical industry. Tissue culture is the best solution for growing plants in large quantities and faster than conventional cultivation. In recent years, diverse bioreactor design has been used for cultivation of differentiated plant organ cultures[11,12]. One of the bioreactor that was used to growing cell and organ culture is balloon-type bubble bioreactor. The application of balloon-type bubble bioreactors has been widely developed for various plant cell and organ culture such as *Morinda citrifolia* (L.) cell culture[13], *Eurycoma longifolia* adventitious root culture[14], *Panax ginseng* CA Meyer hairy root culture[10], *Cyclopia genistoides* (L.) Vent. shoot culture[15], *Hypericum perforatum* shoot culture[16], *Aloe barbadensis*[17], *Dendrobium candidum* Wall ex Lindl. protocorm culture[18], *G. procumbens* adventitious roots culture[19].

Sucrose is a source of energy to build cell organelles that make up plant organs affecting the growth of plant organ cultures and the production of secondary metabolite. Increased levels of sucrose up to 6% could increase biomass of *Talinum paniculatum* hairy root culture[20], 5% sucrose also increased biomass of *G. procumbens* adventitious roots that were grown in liquid culture with agitation[21] and in a temporary immersion bioreactor[22].

The density of explants in bioreactor affected the growth and accumulation of secondary metabolite that was showed in production of ginsenosides from adventitious root suspension cultures of ginseng[23], adventitious roots culture of *Eleutherococcus koreanum*[24] and microtuber production of potato in modified nutrient spray bioreactor system[25]. During the process of culture, explants produced CO_2, which was the result of catabolism process. Increasing the amount of explants will multiply the CO_2 content in the medium, so it affects the growth of explants in the bioreactor[24].

Shoot culture of *G. procumbens* has been done in solid medium and liquid culture in temporary immersion system; the highest growth of shoots was achieved in MS (Murashige and Skoog) solid medium supplemented with kinetin and combination of IAA (indol acetic acid) and BA (benzyl adenine) but in temporary immersion system, number of shoots lower than in solid medium[26]. This study aimed to increase the growth of *G. procumbens* shoots in balloon-type bubble bioreactor (BTBB) influenced by sucrose concentration and inoculums density.

MATERIALS AND METHODS

Gynura procumbens (Lours.) Merr. was obtained from the Botanical Garden Purwodadi, Pasuruan, East Java, Indonesia. This research was conduct at January, 2017 until September, 2018. Stem nodes of *G. procumbens* were used as an explant. Stem nodes sterilization were performed using Clorox 1% (Bayclin, Johnson, Indonesia) for 10 min and rinsed with sterile distilled water three times. The stem nodes were grown in MS[27] supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar and combination of 2 mg L^{-1} IAA and 4 mg L^{-1} BAP. The medium used has been adjusted to pH 5.8 and autoclaved for 15 min (1 atm and 121°C). Cultures were maintained at room temperature (25±3°C) with fluorescent lighting (General electric cool white fluorescent tubes) 1900 lux. On the 3rd day, explants was transferred into MS liquid medium using the same combination of plant growth regulator as solid medium and cultured in a shaker incubator (110 rpm) for 3 days. Explants were ready for use to culture in balloon-type bubble bioreactors.

Shoots culture in bioreactor: Seven shoots as an initial inoculum were grown on 1 L bioreactor using 400 mL of MS liquid medium supplemented with 2 mg L^{-1} IAA and 4 mg L^{-1} BAP with each sucrose concentrations of 10, 30 and 50 g L^{-1}. The inoculums density treatment were done using 5, 10 and 15 shoots explants with 50 g L^{-1} sucrose in each bioreactor. The bioreactor was equipped with an explants retaining wire (net) to reduce hyperhydricity that can interfere explants growth by causing abnormal explants growth[28]. Aeration rate was adjusted to 0.2 vvm. Cultures were maintained by fluorescent light (General electric cool white fluorescent tubes) continuously at 1900 lux and incubation temperature was 25±3°C for 4 weeks.

Data were recorded after 4 week of culture period including total fresh weight, total dry weight, number of shoots per explants, number of leaves per explants and growth index. Dry weight was obtained from the oven-dried shoots at 60°C for 48 h. The pH value and the medium conductivity were measured every week. The growth index of *G. procumbens* shoots was calculated using the following equation[29]:

$$\text{Growth index} = \frac{\text{Final dry weight of planlet} - \text{dry weight of initial inoculum}}{\text{Dry weight of initial inoculum}}$$

Statistical analysis: Biomass of shoots (fresh weight and dry weight), number of shoots, number of leaves and length of shoots data were analyzed using statistical software program (SPSS 19). Each mean value represented the replicate of two

determinations were analyzed using one-way ANOVA test ($p<0.05$). To determine the significant difference between treatments, Duncan's test was done.

RESULTS

Effect of sucrose concentration on shoots multiplication: The highest multiplication of *G. procumbens* shoots in balloon type bubble-bioreactor was obtained in medium supplemented with sucrose 50 gL^{-1}; these results were powered by data of number of shoots/explants, number of leaves, total fresh weight, dry weight and growth index (Table 1). The higher of sucrose concentration in medium, more the number of shoots per explants formed. The highest length of shoots were achieved by treatment of 10 g L^{-1} sucrose followed by sucrose treatment of 50 and 30 g L^{-1}, respectively. The length of shoot was almost the same in the treatment of 10 and 50 g L^{-1} sucrose. Morphology of *G. procumbens* shoots in various concentration of sucrose was showed at Fig. 1a-c. Although the number of shoots and number of leaves in the treatment of 10 and 30 g L^{-1} sucrose were not significantly different, the length of shoots produced was significantly different.

Effect of initial inoculums density on shoots multiplication: The initial inoculums density affected the growth of *G. procumbens* shoots in balloon-type bubble bioreactors (Fig. 1d-e). The fastest shoot multiplication was obtained in the treatment of inoculums density 5 explants, that produced 13.8 shoots/explants and number of leaves 272.2 but length of shoots were not significantly different with the other treatments. Fresh weight and dry weight increased along with the increasing inoculums density planted. The highest growth index was achieved at the treatment of inoculums density 5 explants but the highest length of shoots was obtained in inoculums density 15 explants, although based on statistical analysis was not significantly different (Table 2).

Physical condition of medium: The physical condition of medium during shoots culture in balloon type bubble bioreactors was showed by pH values and electrical conductivity. The pH of the medium had a tendency to decrease during 4th week culture period (Fig. 2). In the treatment of various sucrose concentrations, the decrease of pH at the end of culture was not significantly different, that was 4.6-4.8 but in the treatment of 5 explants as inoculums density, there was a significant decrease of pH at the end of culture, which was 4. The decrease in pH was most prevalent in the inoculums density of 15 explants. The available nutrition in the medium can be utilized for plant growth. This was supported by the pH and electrical conductivity of the medium that decreased gradually from week to week. In the treatment of 5 explants, the electrical conductivity of medium increased from week to week although it is not significant (Fig. 3).

The electrical conductivity of the medium decreased until the end of the 4th week of culture, except in the initial inoculums density of 5 explants. In the treatment of 5 explants, the medium conductivity showed an increase but not significant (Fig. 3).

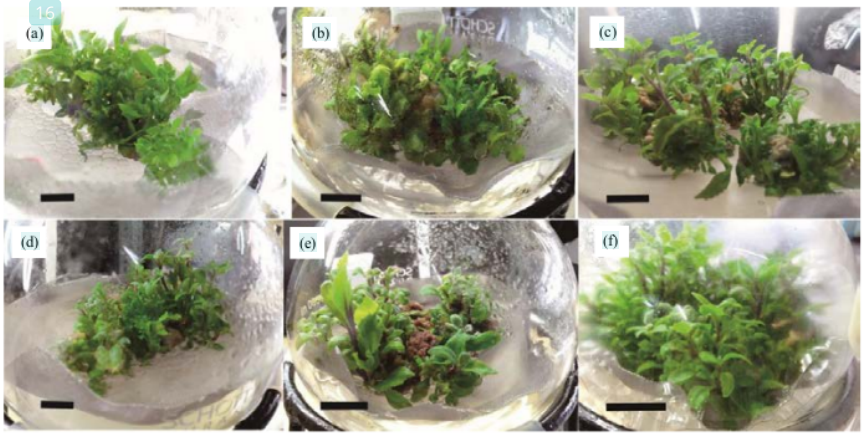

Fig. 1(a-f): Shoot culture of *G. procumbens* in BTBB, (a) Sucrose treatment 10 g L^{-1}, (b) 30 g L^{-1}, (c) 50 g L^{-1} and inoculums density (d) 5 explants, (e) 10 explants and (f) 15 explants
Bars: 1.4 cm

Table 1: Effect of sucrose concentrations on multiplication of *G. procumbens* shoots culture in balloon type bubble-bioreactor

Sucrose concentration (g L^{-1})	No. of shoot/explants	No. of leaves	Length of shoots (cm)	Total FW (g)	Total DW (g)	Growth index
10	4.00±1.57^{b}	151.21±49.71^{ab}	4.34±1.10^{a}	16.85	0.46	0.93
30	5.64±3.27^{b}	118.36±68.32^{ab}	3.15±1.16^{b}	22.94	1.22	4.10
50	9.36±5.050^{a}	195.50±71.5^{a}	4.03±0.95^{ab}	26.95	2.10	7.80

Mean values within a column followed by the same letters are not significantly different at p = 0.05 according to Duncan's Multiple Range test

Table 2: Effect of inoculums density on multiplication of *G. procumbens* shoots cultured in balloon type bubble-bioreactor

Inoculums density (explants)	No. of shoot/explants	No. of leaves	Length of shoots (cm)	Total FW (g)	Total DW (g)	Growth index
5	13.80±2.39^{a}	272.20±109.94^{a}	3.94±0.40^{a}	17.79	0.94	12.80
10	7.60±3.27^{b}	102.80±24.68^{c}	3.89±0.49^{a}	27.39	2.23	6.60
15	6.67±3.85^{b}	178.33±73.17^{b}	4.94±1.23^{a}	49.09	3.13	5.60

Mean values within a column followed by the same letters are not significantly different at p = 0.05 according to Duncan's Multiple Range test

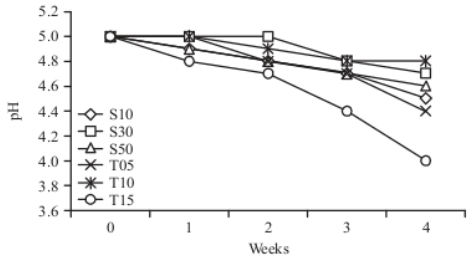

Fig. 2: The pH of medium during *G. procumbens* shoot culture period in balloon-type bubble-bioreactor
S10: 10 g L^{-1} sucrose, S30: 30 g L^{-1} sucrose, S50: 50 g L^{-1} sucrose, T05: 5 explants, T10: 10 explants, T15: 15 explants

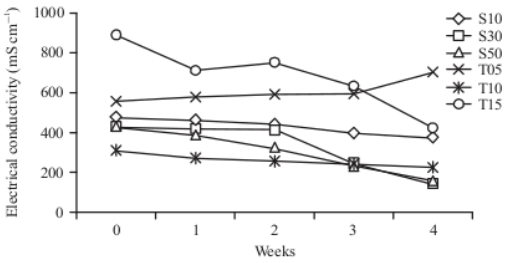

Fig. 3: Electrical conductivity medium during *G. procumbens* shoot culture period in balloon type bubble-bioreactors
S10: 10 g L^{-1} sucrose, S30: 30 g L^{-1} sucrose, S50: 50 g L^{-1} sucrose, T05: 5 explants, T10: 10 explants, T15: 15 explants

DISCUSSION

The higher sucrose concentration supplemented in the culture medium, the higher carbon source was available in the medium, so it increased the multiplication of shoots. Sucrose will be hydrolyzed into free glucose and fructose by invertase enzyme before being used[30]. Some of the sucrose in the tissue culture medium is hydrolyzed into free fructose and glucose during the process of sterilization[31]. Hydrolyzed sucrose is easier used by the plant for the process of cell metabolism and cell organelles builders. Sucrose also plays an important role in the formation of secondary metabolite compounds used as medicinal ingredients[32]. In this study higher sucrose concentration in medium could enhance shoot multiplication. Optimum results were also obtained at higher sucrose addition in *A. barbadensis* shoot culture[17], *Talinum paniculatum* adventitious root[20] and *Dendrobium candidum* protocorm[18].

The number of shoots increased along with the concentration level of sucrose. That was also reported in *A. barbadensis* shoot culture[17]. It indicated that the availability of high sucrose in the medium is utilized well for the growth process. Sucrose also affected the growth index of *G. procumbens* shoots in this study. Sucrose not only acts as a carbon source for build plant organs but also has a function as osmotic control of plant tissues[30]. The difference in concentration gradient between plant cells will affect the polarity of plant cells that will affect the morphogenesis process and the growth index of the plant. However, the concentration gradient difference is not the only factor affecting the polarity process that affects plant morphogenesis. There are other factors that affect the process of plant morphogenesis, such as genetic factors of plants[32].

The number of shoots obtained was getting lower along with the higher inoculums density planted. The highest number of leaves were obtained at the treatment of initial inoculum of 5 explants. The more number of initial inoculums, the tighter competition to get nutrition and space to grow. The tight competition affected the growth of plants. It was also reported on the production of potato microtubers[25]. In this study the best shoot height was obtained at the density treatment of 15 explants. This phenomenon proved that more explants planted, the plants will compete to get the light so that trigger the plant to grow higher towards the light to support the process of photosynthesis. But, the

treatment of 5 explants and supplementation of sucrose 50 g L^{-1} was the best treatment to obtain the high multiplication of shoots (13.8 shoots/explants and 9.38 shoots/explants). This result showed that *G. procumbens* shoots cultured in balloon-type bubble-bioreactor increased multiplication of shoots better than in solid medium or in temporary immersion system. In solid medium, maximum number of shoots was 7 shoots/explants, whereas in temporary immersion system was 8 shoots/explant[26].

The total fresh weight and total dry weight increased along with the high inoculums density but the growth index was decreased. Cui *et al.*[18] reported that fresh weight and dry weight were higher along with the higher inoculums density grown in bioreactors. Lee *et al.*[24] also reported that fresh weight and dry weight obtained were higher and the growth index obtained was lower along with the high inoculums density on the product ion of *Eleutherococus koreanum* Nakai root in bioreactors. In this study, the best dry weight was obtained in the initial inoculums treatment of 15 explants. This proved that the plants formed the cell mass well, so the percentage of moisture content in the cell was the lowest compared to other treatments.

CONCLUSION

From the results obtained, it can be inferred that supplementation of 50 g L^{-1} sucrose produce the highest number of shoots and growth index of *G. procumbens* shoot culture, whereas the highest number of shoot and growth index was obtained at inoculums density 5 explants.

SIGNIFICANCE STATEMENT

This study discovered the shoot multiplication of *G. procumbens* in balloon-type bubble bioreactor can increased significantly, so that this method can be beneficial to produce biomass in large scale. This study will help the researchers to uncover the critical areas of shoot multiplication in bioreactor that many researchers were not able to explore. Thus a new method to speed up micropropagation of this plant maybe arrived at to support the need of pharmaceutical industry.

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