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Effect of sucrose and potassium nitrate on biomass and saponin content of *Talinum paniculatum* Gaertn. hairy root in balloon-type bubble bioreactor

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ABSTRACT

Objective: To increase biomass and saponin production in hairy root culture of *Talinum paniculatum* Gaertn. (*T. paniculatum*) in balloon-type bubble bioreactor (BTBB).
Methods: Hairy roots which were collected from leaf explants of *T. paniculatum* were infected by *Agrobacterium rhizogenes* strain LB510. The hairy roots were cultivated at 400 mL Murashige and Skoog liquid medium without growth regulator (MS0) in 1 000 mL BTBB. Each BTBB had 2 g hairy roots as initial inoculum and these cultures were treated with various concentrations of sucrose (3%, 4%, 5%, 6% w/v) and potassium nitrate (0.5, 1.0, 1.5 and 2.0 strength of MS medium). Cultures were maintained for 14 days. Fresh and dry weights of hairy roots at the end of culture were investigated.
Results: Various concentrations of sucrose influenced the biomass accumulation of hairy roots. Maximum biomass was reached by MS medium supplemented with 6% sucrose and it was approximately threefold higher than control. Culture supplemented with potassium nitrate at 2.0 strength of MS0 could increase biomass accumulation of hairy roots until 0.14 g dry weight and it was almost threefold higher than control. However, the maximum saponin content was obtained by MS medium supplemented with 5% sucrose and 2.0 strength potassium nitrate of MS.
Conclusions: Based on this research, those conditions can be used to produce biomass and saponin of hairy root of *T. paniculatum* in the large scale.

1. Introduction

Java ginseng [*Talinum paniculatum* Gaertn. (*T. paniculatum*)] has been used in pharmaceutical industries for source of saponins, flavonoids, tannins, triterpenes or sterols, and polyphenols. Saponins of *T. paniculatum* are accumulated in roots. Ability and effectiveness of saponins on many medicinal treatments have been scientifically proven. Saponins were reported to be able to enhance viability, motility and number of spermatozoa. Saponins also act as a anti-inflammatory agent, have androgenic potency, are able to induce cell differentiation through receptor cells [1], and could increase body resistance to disease [2]. *T. paniculatum* needs 3–4 years to produce the maximum saponins in the root. Root culture technology could be a solution to fill saponins demand in the market. This technology is important to be developed for plant preservation and increasing saponin content in roots.

Transformation by using *Agrobacterium rhizogenes* (*A. rhizogenes*) as a mediator to transfer transfer-DNA (T-DNA) into plant DNA is shortly alternative to produce roots. The T-DNA contains genes encoding enzymes for the synthesis of the phytohormones cytokinin and auxin, and of specific opine. The expression of oncogenes in Ri plasmid is indicated by adventive roots formation in infected area of explants. These adventive

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roots are called hairy roots. In recent decades, hairy root culture has been widely used to produce various types of secondary metabolites that are naturally present in the roots. It was previously reported that *rolC* from *A. rhizogenes* T-DNA was shown to stimulate the production of secondary metabolites in the transformed plant cells of different plants. It was revealed that hairy roots enhanced the amount of glycyrhizin in *Glycyrrhiza glabra* [3], plumbagine in *Plumbago rosea* [4], saponin in *Bacopa monnieri* [5], anthraquinones in *Polygonum multiflorum* [6], and polyphenols in *Momordica charantia* [7]. Organic nutrients in plants play a role in growth, development and accumulation of secondary metabolites. Growth and synthesis of secondary metabolite in hairy roots are also influenced by the nutritional quality. The effect of sucrose and nitrate concentrations in culture medium had been investigated. Biomass growth and withanolide A, production of *Withania somnifera* hairy roots were affected by different carbon sources in the Murashige and Skoog liquid medium (MS medium) [8]. Biomass and metabolite accumulation were also affected by NH_4: NO_3 ratio in balloon-type bubble bioreactor (BTBB) culture of *Eurycoma longifolia* adventitious roots [9].

In previous studies, hairy roots of *T. paniculatum* that were cultivated in solid MS medium without growth regulator (MS0 medium) grew slowly. Hairy root growth was accelerated after subculture in semi-solid MS0 medium, but limited by oxygen supply and space growth. Therefore, hairy roots must be subcultured to liquid medium. Liquid culture has some advantages, for example, oxygen demand is filled by agitation or aeration, culture space limitation is solved by widening bioreactor chamber, pH medium is under control, nutrients are more homogenized and available for all parts of explants. Culture of *T. paniculatum* hairy roots in BTBB has mainly focused on aeration rates and inoculum densities. The best aeration rate and inoculum density for biomass and saponin content were reached at 2 g/400 mL and 0.25 vvm respectively [10]. In this research, various concentrations of sucrose and nitrogen are determined to increase biomass and saponin content.

2. Materials and methods

2.1. Materials

T. paniculatum was obtained from Botanical Garden of Purwodadi, Indonesia. *T. paniculatum* leaves were used as explants to initiate hairy roots. *A. rhizogenes* LB510 was infected to *T. paniculatum* leaves to induce hairy roots. The bacteria were obtained from Research Center of Biotechnology, Indonesian Institute of Sciences, Indonesia.

2.2. Explant sterilization and induction of hairy roots

The leaf explants of *T. paniculatum* were washed briefly with detergent and rinsed with running tap water. After rinsing, the explants were sterilized with 10% (v/v) clorox for 5 min, and then rinsed 3 times with sterile water. After sterilized and rinsed, the explants were shaken gently. The sterile leaf explants were cut ± 1 cm^2 and ready to be infected by *A. rhizogenes* LB510. The bacteria were grown in liquid Luria-Bertani medium at 110 r/min, and (28 ± 2) °C for 2 days. The suspension of *A. rhizogenes* LB510 was diluted with liquid MS0 medium, then 100 μmol/L acetosyringone was added. The explants were submerged in the solution for 5 min and were shaken gently. After that, the explants were drained on sterile filter paper and then transferred to MS0 agar. The explants were incubated at (28 ± 2) °C under dark condition for 2 days. In the next step, the explants were transferred to MS0 solid medium supplemented with 500 mg/L cefotaxime. The explants were incubated at (28 ± 2) °C under dark condition for a week, and then were moved into MS0 semi-solid medium (5 g/L agar) for a week. Successful transformation was known with hairy root formation from the edge of the leaf explants. In the end of incubation time, hairy roots were excised from explants and then transferred to 250 mL Erlenmeyer flask. The flask contained 50 mL liquid MS0 supplemented with 500 mg/L cefotaxime. Hairy roots cultures were agitated at 90 r/min, (28 ± 2) °C, under dark condition for a week. At the end of culture, hairy roots were ready to use as inoculum on liquid culture in BTBB.

2.3. Liquid culture condition in BBTB

Volumes of BBTB vessel were 1 000 mL with working volumes between 200 and 500 mL. About 400 mL of MS0 medium was placed in 1 000 mL Erlenmeyer flask and sterilized on autoclave at 121 °C for 20 min. Liquid MS0 medium was transferred into BTBB aseptically. Initial inoculum was 2 g hairy roots and all BTBB cultures were aerated at flow rate of 0.25 vvm.

2.4. Treatment of sucrose and potassium nitrate at various concentrations

These experiments had eight BTBB cultures. Each culture was in the same conditions, such as 2 g hairy roots as inoculum, 0.25 vvm of air flow rate and 400 mL volume of MS0 medium. In this test, eight BTBB were treated with various concentrations of sucrose [3%, 4%, 5%, 6% (w/v)] and various concentrations of potassium nitrate (0.5, 1.0, 1.5 and 2.0 strength of MS medium). Cultures were incubated under dark condition at 25 °C for 14 days. The changes of pH, conductivity and total sugar in culture medium were checked every two days. Conductivity and total sugar content in medium culture were checked with hand conductometer (Ezdo) and hand refractometer (Atago). At the end of cultivation, biomass and saponin content were measured.

2.5. Saponin analyses

Saponin content of hairy roots was analyzed qualitatively by using thin layer chromatography and quantitatively by using high performance liquid chromatography (HPLC). Hairy roots were dried at 50 °C for 5 days and then were ground with mortar. About 100 mg powders of hairy roots were immersed in 10 mL ethanol, and then heated at 80 °C in water bath for 30 min. The hairy root extract was concentrated at 80 °C for 3 h until a volume of 0.2 mL. Extract and standard saponin (Calbiochem) were spotted on silica gel GF254 and were eluted by using eluent propanol: water (14:3). Spot was detected by spraying with anisaldehyde 0.5 mL, acetic acid glacial 10.0 mL, ethanol 85.0 mL, and sulfuric acid 5.0 mL and then heated at 110 °C for 6–10 min. Standard saponin will be dark green color.

Quantitative measurement of saponin was analyzed by using HPLC system (Agilent Q-TOF 6530 L) equipped with C18 columns. The mobile phase was mixtures of 0.1% formic acid in water grade and acetonitrile (40:60, v/v), and the injection volume was 0.2 µL. The wavelength of detection was set at 299 nm.

3. Results

3.1. Induction of hairy roots

The hairy roots of *T. paniculatum* were transformed successfully by *A. rhizogenes* LB510 as shown in Figure 1A–C. Hairy roots as inoculum on liquid culture in BTBB were shown in Figure 1D. In the preliminary study, we did the transformation with two strains of *Agrobacterium*, *A. rhizogenes* LB510 and YMB072001, with different durations of infection and different explants (leaf and stem). The highest transformation efficiency was obtained by *A. rhizogenes* strain LB510 with 5-min infection at bacterial concentration and OD600 = 0.1 (78.43%), whereas for *A. rhizogenes* strain YMB072001, the highest transformation efficiency was obtained in 10-min infection (71.47%). Meanwhile, transformation by two strains of *A. rhizogenes* leaf explants obtained higher transformation efficiency than by stem explants. In *A. rhizogenes* strain LB510, transformation efficiency was 73.33% (leaf explants) and 66.67% (stem explants), whereas in *A. rhizogenes* strain YMB072001, transformation efficiency was 60.00% (leaf explants) and 56.67% (stem explants).

3.2. Effects of sucrose at various concentrations on biomass and saponin content

Sucrose at different concentrations was tested as carbon source for hairy root growth of *T. paniculatum* in BTBB. The results showed that different concentrations of sucrose provided different growth and saponin content in each hairy root culture (Figure 2). Hairy root culture with supplemented sucrose 6% had the maximum dry weight. The saponin content of *T. paniculatum* hairy roots which were cultured in medium supplemented with sucrose at range 3%–5% was higher than in medium supplemented with 6% sucrose. In this research, the maximum saponin content reached 71365 mg/L/g dry weight in the medium supplemented with 5% sucrose (Figure 2 and Table 1).

Detection of hairy root growth of *T. paniculatum* in various concentrations of sucrose was done by measurement of total sugar, pH and electric conductivity (EC) medium during 14-day cultivation (Figure 3). Total sugar of all treatments at different sucrose concentrations in BTBB for 14 days showed the decrease trend (Figure 3A).

In this research, the uptake of macronutrients and micronutrients by hairy roots was shown by changes of EC medium. During the first week, the EC value in all culture medium decreased gradually (Figure 3C). The biggest changes of total sugar and EC values were obtained in medium culture supplemented with 5% sucrose and the changes indicated the ability of hairy roots to absorb sucrose, macronutrients and micronutrients. During the cultivation, the pH value of all culture medium decreased (Figure 3B).

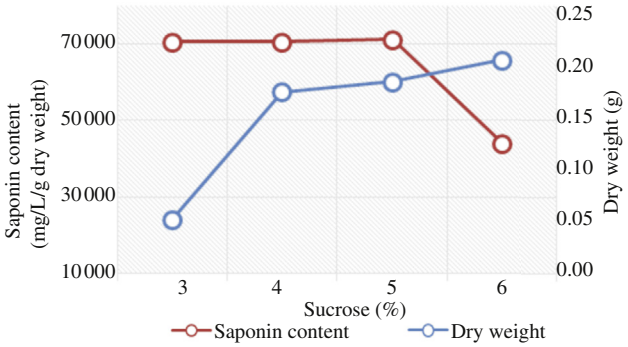

Figure 2. Biomass and saponin content of *T. paniculatum* hairy roots in various concentrations of sucrose after 14-day culture.

Table 1

Saponin content of *T. paniculatum* hairy roots in various concentrations of sucrose and potassium nitrate for 14 days.

Treatment		Saponin (mg/L/g dry weight)
Sucrose concentration (%)	3.0	70717
	4.0	70717
	5.0	71365
	6.0	44030
Potassium nitrate strength (×MS)	0.5	63299
	1.0	70717
	1.5	70726
	2.0	70730

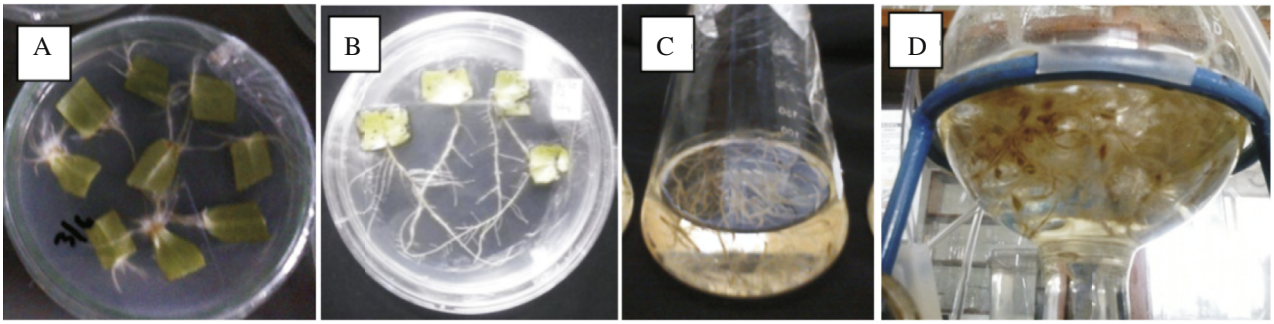

Figure 1. Induction of *T. paniculatum* hairy roots in MS0 medium.
A: 9 days old in solid medium containing 500 mg/L cefotaxime; B: 16 days old in semi-solid medium containing 500 mg/L cefotaxime; C: 23 days old in liquid medium containing 500 mg/L cefotaxime; D: 30 days old as inoculum in liquid culture in BTBB.

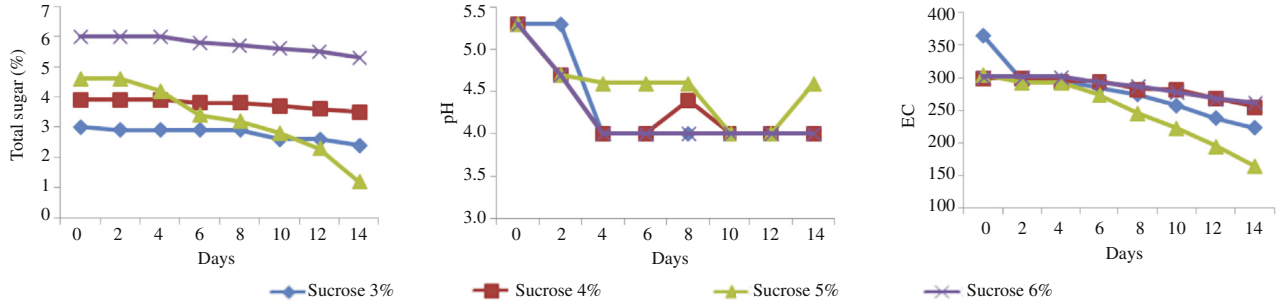

Figure 3. Total sugar content, pH, and EC of culture medium of *T. paniculatum* hairy roots during 14 days cultivation in various concentration of sucrose.

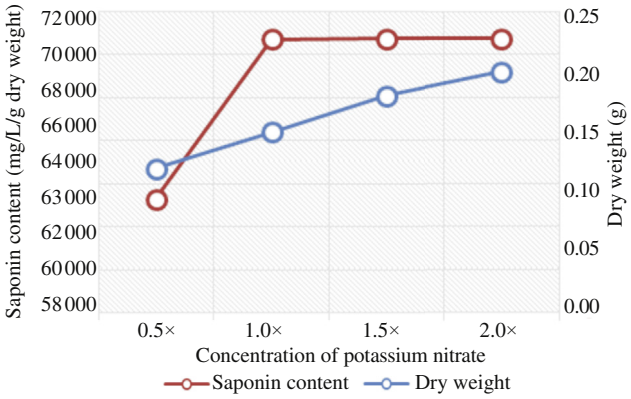

Figure 4. Biomass and saponin content of *T. paniculatum* hairy roots at various concentrations of potassium nitrate after 14-day culture.

3.3. Effect of potassium nitrate at various concentrations on biomass and saponin content

The hairy root biomass of *T. paniculatum* at various potassium nitrate concentrations is shown in Figure 4. Supplement with potassium nitrate at 1–2 strength of MS medium could increase hairy root biomass after 14-day cultivation. The biomass and saponin content were increased at potassium nitrate 1–2 strength.

Hairy root growth of *T. paniculatum* was shown by profile of total sugar, pH, and EC medium during 14-day cultivation (Figure 5). Total sugar and EC values from the start till the end of culture showed down-trend line (Figure 5A and 5C). The saponin content of *T. paniculatum* hairy roots was analyzed by two methods. The results of qualitative and quantitative test by

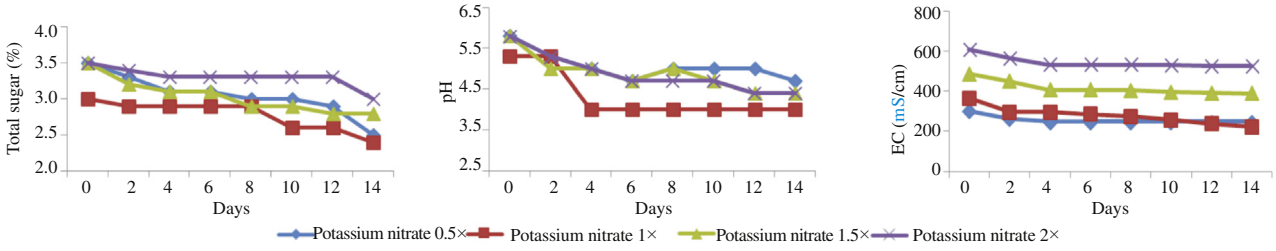

Figure 5. Profile of total sugar content, pH and EC medium of *T. paniculatum* hairy roots during 14-day cultivation in various concentrations of potassium nitrate.

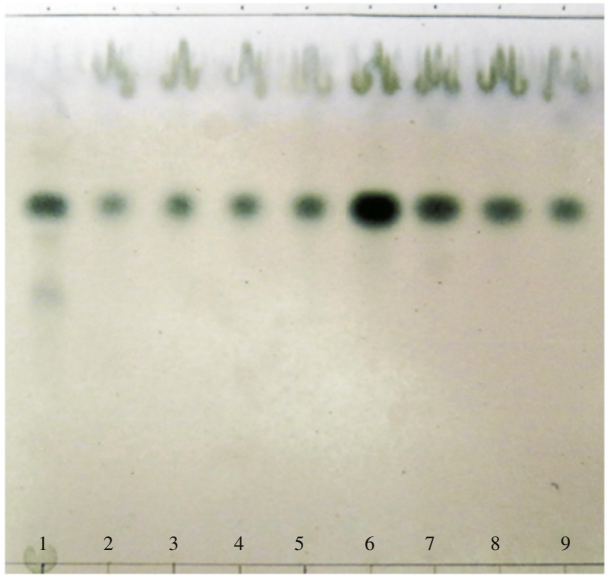

Figure 6. Chromatogram of ethanol extract of *T. paniculatum* hairy roots after cultured in 1 000 mL BTBB containing 400 mL MS medium with various concentrations of sucrose and potassium nitrate for 14 days.
1: Saponin standard; 2: At 3% of sucrose w/v; 3: At 4% of sucrose w/v; 4: At 5% of sucrose w/v; 5: At 6% of sucrose w/v; 6: At 1 potassium nitrate strength of MS medium; 7: At 0.5 potassium nitrate strength of MS medium; 8: At 1.5 potassium nitrate strength of MS medium; 9: At 2 potassium nitrate strength of MS medium.

using thin layer chromatography and HPLC are shown in Figure 6 and Table 1 respectively.

4. Discussion

Sucrose is the source of carbon that is widely used in cell culture of plants, animals, fungi and bacteria. Cells use sucrose for energy and biosynthesis, including the biosynthesis of secondary metabolites. The maximum absorption of sucrose and the other nutrients increased dry weight and saponin content of hairy roots that were cultivated in medium supplemented with 5% sucrose (Figure 2).

Ability of *T. paniculatum* hairy roots to consume sucrose, macronutrients and micronutrients had correlation with pH medium. The initial pH medium was around 5.3 after autoclaving. The cultures supplemented with 3%, 4% and 6% sucrose underwent a little changes of total sugar content and EC value during cultivation. These changes correlated with pH value of culture medium. Decrease of pH in culture medium supplemented with 3%, 4% and 6% sucrose was drastical and it begun on the second day of cultivation. The pH medium was changed from 5.3 to 4.0, so the culture medium became too acid for hairy root growth. Acidity of medium did not cause the maximization of absorption of sucrose, macronutrients and micronutrients. Contrary with the other cultures, the change of

total sugar content and EC value in medium supplemented with 5% sucrose was bigger than medium supplemented with 3%, 4%, and 6% sucrose. It was also correlated with pH changes in medium during cultivation. The pH changes of medium supplemented with 5% sucrose were not drastical like the others. The pH value around 4.5–5.0 in medium was begun from the second day till the end of culture, so the absorption of sucrose, macronutrients and micronutrients was maximum. Therefore, growth of hairy roots was enhanced until 2 weeks. It is shown that hairy roots cultured were at exponential phase. This phenomenon was supported by the earlier study which reported that adventitious root growth typically exhibited a lag phase from 0 to 1 week, an exponential phase from 1 to 5 weeks, a stationary phase from 5 to 6 weeks, and a declining phase thereafter [11]. Concentrations of sucrose also effected on growth and saponin content of *T. paniculatum* hairy roots. Sucrose at concentrations of 3%–6% in culture medium was able to increase dry weight and saponin content of *T. paniculatum* hairy roots, but 5% sucrose was known as the best concentration of sucrose to enhance dry weight and saponin content. The effect of sucrose concentrations on growth and production of withanolide A was also reported on *Withania somnifera* hairy root culture. Among the various concentrations of sucrose (1%–8% w/v) tested, 3% sucrose favored the highest accumulation of biomass, whereas 4% sucrose concentration favored the highest production of withanolide A [9]. MS liquid medium containing sucrose 3% with an initial pH 5.8 favored the maximum biomass accumulation of *Solanum trilobatum* L. hairy roots [12].

Furthermore, hairy root culture of *Glycyrrhiza inflata* in MS medium with various concentrations of sucrose obtains the maximum biomass and glycyrrhizin in MS medium containing 12% and 6% sucrose, respectively [13]. Concentration of sucrose in medium also influenced the biomass production of *Artemisia vulgaris* hairy roots. Biomass accumulation was the highest when the medium was supplemented with 40 g/L sucrose [14].

Nitrogen is an essential element that composes most plant body, such as 1.5%–2.0% of dry weight of plant body and around 16.0% of total protein of plant body. In the cytoplasmic roots, nitrate as a nitrogen source is saved in vacuole and transported by xylem to all around the plant body. Nitrate is also as a key enzyme in amylum biosynthesis pathway, so that the carbon bone for amylum is shifted to carbon bone for nitrogen assimilation. The changes of amylum biosynthesis pathway to nitrogen assimilation pathway will increase proteins and enhance the plant growth rate. Generally, biomass growth rate has a direct correlation to secondary metabolite contents. All treatments of various concentrations of potassium nitrate enhanced dry weight and saponin content of *T. paniculatum* hairy roots. It indicated that the hairy roots were still in growth during cultivation of 14 days. In this research, the activity of hairy roots to absorb potassium nitrate for biomass growth and saponin formation was proved by decreasing trend line of EC curve. Enhancement of potassium nitrate from 950 mg/L (0.5 strength) to 3 800 mg/L (2.0 strength) influenced biomass and saponin content of hairy roots of *T. paniculatum*. Dry weight and saponin content tended to increase with increasing concentrations of potassium nitrate (Figure 4 and Table 1).

Various concentrations of potassium nitrate in this research also affected the ratio of ammonium to nitrate and differentiation of total nitrogen concentration in each culture medium. However, the results of dry weight and saponin content of *T. paniculatum* hairy roots proved that the ratio of ammonium to nitrate and different concentrations of total nitrogen influenced the production of secondary metabolites. This phenomenon was also reported in another production of secondary metabolites. The accumulation of secondary metabolites in the adventitious roots of *Eurycoma longifolia* was affected by NH_4^+: NO_3^- ratio. The secondary metabolites increased with NH_4^+, and the highest levels were obtained when NH_4^+ was the sole nitrogen source, which was contrasted with the root growth results [9]. Productivity of biomass and bioactive compounds through bioreactor culture was also affected by medium salt strength, such as accumulation of bioactive compounds in the adventitious roots of *Eleutherococcus koreanum* Nakai, which indicated that $^1/_2$ MS was the optimal salt strength for the production of both biomass and bioactive compounds [15].

Various concentrations of sucrose in liquid culture by using MS0 medium could influence growth of hairy roots of *T. paniculatum*. The maximum biomass was reached in culture supplemented with 6% sucrose and approximately three fold higher than control. Culture supplemented with potassium nitrate at 2.0 strength of normal MS0 medium could increase dry weight of hairy roots until almost threefold higher than control. However, the maximum saponin content was obtained in culture supplemented with 5% sucrose and 2 strength potassium nitrate of normal MS0. Based on this research, those conditions can be used to produce biomass and saponin of hairy root of *T. paniculatum* in the large scale.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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