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Original Research Article

Biomass and Flavonoid Production of *Gynura procumbens* (Lour.) Merr. Axillary Shoots Culture Induced by Sucrose and Erythrose 4-Phosphate

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Abstract: *Gynura procumbens* (Lour.) Merr. is a medicinal plant which was used in Indonesia, Malaysia, Thailand, and another region of South East Asia. These plants contain many compounds which useful as source of medicine like flavonoid. In order to increase biomass and flavonoid production, in vitro axillary shoots culture were initiated from stem node. Axillary shoot were cultured in Murashige and Skoog (MS) medium supplemented with 2 mg/L indol acetic-acid, 4 mg/L benzyl adenine, sucrose (10, 30, 50 g/L), and erythrose 4-phosphate (0, 1, 2.5, 5, 25 µM). Results showed that the highest fresh weight was achieved in medium supplemented with sucrose 50 g/L and erythrose 4-phosphate 5 µM, whereas supplemented of sucrose 30 g/L and erythrose-4-phosphate 5 µM gave the highest dry weight. Increasing supplementation of sucrose will increase cathecine content in all treatment. The highest concentration of quercetin and kaempferol was achieved in medium supplemented with sucrose 30 mg/L and erythrose 4-P 1 µM.
Keywords: Plant tissue culture, Biomassa, Erythrose 4-phosphate, Flavonoid, Axilary shoot culture, *Gynura procumbens*.

INTRODUCTION

Gynura procumbens is an important medicinal plant in tropical region, especially in Indonesia, Malaysia, and Thailand because this plant has been long used as a vegetable and medicinal plant. Leaves of this plant were used for many diseases that are caused by oxidative stress, such as inflammation, diabetes, cancer, and hypertension [1]. As a medicinal plant *G. procumbens* have potential bioactive compound such as flavonoid, saponin, alkaloid, tannin, terpenoid and sterol glycoside [2]. Flavonoid have many function in plant tissues, such as produce the pigment that protected plant from UV-B, activator of gene that induced nodulation, and as a phytoalexin. Beside that, flavonoid also have important role for human in food and pharmaceutical industry [3]. Previous study indicated that flavonoid believed as one of phenolic compound that have anti oxidative character and prevent cell damaged from free radical compound.

Supplemented of precursor in culture medium was the approach to induced secondary metabolite production. Precursor was an intermediate compound in secondary metabolism pathway; supplementation of precursor in culture medium could increase the yield of certain compound. Supplementation of precursor such as phenylalanine could increased taxol production in cell culture of *Taxus cuspidata* [4] and quercetin production in cell culture of *Citrullus colocynthis* (Linn.) Schrad [5]. Cynnamyl alcohol which was supplemented in medium of callus culture *Rhodiola rosea* could induced production of cinnamyl glycoside [6]. Flavonoid production also could increased with supplementation of methyl jasmonate in cell suspension culture of *Taxus chinensis* var. Nairei [7] and chitosan in callus culture of *Glycyrrhiza glabra* [8].

Part of biosynthesis pathway of flavonoid in plant initiated from phenylpropanoid (shikimate pathway), which was also found in other secondary metabolites such as alkaloid, lignin, suberin, and monolignole. First step in shikimate pathway was condensation of erythrose 4-P from pentose phosphate pathway [9]. Many research showed that genes encode enzymes which were involved in phenylpropanoid pathway could influenced by source of carbohydrate, such as glucose and sucrose that were role as signal molecule. Beside that, sucrose also provided carbon source for secondary metabolite and biomass production [10, 11]. In previous study we found that supplemented of sucrose 50 g/L

could increased biomass of adventitious roots of *G. procumbens* and induced formed of isoflavon (group of flavonoid) in temporary immersion system. In recent years, organ culture of *G. procumbens* was still limited, especially shoot culture. Therefore, developing of other organ culture was necessary to provide *G. procumbens* plants material for use as source of pharmaceutical industry. This research was conducted to know the effect of sucrose and erythrose 4-P as a precursor of flavonoid on production of biomass and flavonoid content of in vitro axillary shoot culture of *G. procumbens*.

MATERIALS AND METHODS

Experimental material

G. procumbens was obtained from florist in Surabaya, East Java, Indonesia. The plant species was identified and confirmed by Botanical Garden Purwodadi, Indonesian Institute of Science, Pasuruan, East Java, Indonesia.

Induction of axillary shoots

Stems of *G. precumbens* approximately 5 cm from shoot were cut and washed with detergent and then were rinsed with tap water. Stems segment were cut in internode part (0.5-1.0 cm) and then surfaced sterilized with clorox 20% (v/v) containing 5.25% sodium hypochloride for 10 min and continue rinse with sterile distilled water. Stems then planted in MS (Murashige and Skoog) solid medium supplemented with IAA (indolacetic acid) 2 mg/L, BA (benzyladenine) 4 mg/L and various concentration of sucrose (10, 30, 50 g/L) and erythrose 4-P (0, 1, 2.5, 5, 25 µM). Stem cultures were incubation at 25 ± 2ºC under continuous illumination. After six weeks old, axillary shoots were harvested and then were dried in the oven at 60ºC until constant weight (DW) was attained.

Determination of growth curve

In order to determine of axillary shoots age which would be harvested; we have to determine of growth curve. Five stems segment were planted in MS solid medium supplemented with 2 mg/L IAA, 4 mg/L BA and 30 g/L sucrose. Measurement of axillary shoots growth were done every 7 days for 56 days. Growth parameters of axillary shoots were fresh weight, dry weight, shoot length, and number of shoot.

Extraction of flavonoids

Dry axillary shoots were pondered 0.05 g and grinded with mortar until formed powder. The powder was extracted by 10 mL ethanol in 60ºC for 5 min, and then was maseration for 24 hours. Extract were filtered and concentrated until 2 mL. Ethanol extract were taken 900 µL and added 100 µL aquadest so the final volume of 90% ethanol extract was 1 mL, then were added 1 mL n-hexane and homogenized with vortex until formed two layers. The lower layer was taken for next analysis.

Determination of total flavonoids

Ethanol extract of flavonoid which was separated by n-hexane were taken 10 µL and spotted on silica gel 60 F_{254} (Merck) and eluted using ethyl acetate: methanol (4:1). Spots were analyzed by UV light at 366 nm. Total flavonoid content was assayed by UV colorimetric [12]. Sampel of each treatment was taken 0.25 mL, and then were added 1.25 mL aquadest and 75 µL $NaNO_2$ solutions. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and incubation for 5 min. Extract were then added 0.5 mL 1 M NaOH and aquadest until volume of solution 25 mL. Absorbance of the mixed solution was measured at 510 nm by UV-Vis spectrophotometer (BOECO S-22, Germany). Cathechin, quercetine, and kaempferol were used as standard compound for the quantification of total flavonoid.

RESULTS AND DISCUSSION

Growth curve of axillary shoots

Results (Figure 1) showed that based on fresh weight, growth of axillary shoots occurred at 7 days culture to 42 days and became stationer at day 49, but based on dry weight, stationer phase occurred at day 42. Shoot length still growth until the end of experiment, but number of shoots has not already increased after 28 days. In this research, harvested of axillary shoots was done when culture came in the early stationery phase. According to Agostini-Costae et al. [13], highest production of secondary metabolite occurred at transition between growth phase to stationer phase, so in this research harvested of axillary shoot was done at day 42.

Effect combination of sucrose and erythrose 4-P on biomass production

Research results showed that the highest fresh weight was found in combination treatment of sucrose 50 g/L and erythrose 4-P 5 µM and its treatment has significant different with others treatment on Games-Howel test (Table 1). Based on dry weight data, the highest biomass was found in combination treatment of sucrose 30 g/L and erythrose 4-P 5 µM, whereas the lowest of fresh and dry weight was found in combination treatment sucrose 10 g/L without erythose 4-P. Measurement of fresh weight was depended on content of water in shoots. Different of fresh weight was caused by different capability of plant cell to storage water and mineral nutrition, so measurement of fresh weight less accurate to show the growth of plant because the data was fluctuate depended on plant moisturize. Measurement of plant biomass more accurate was used by dry weight.

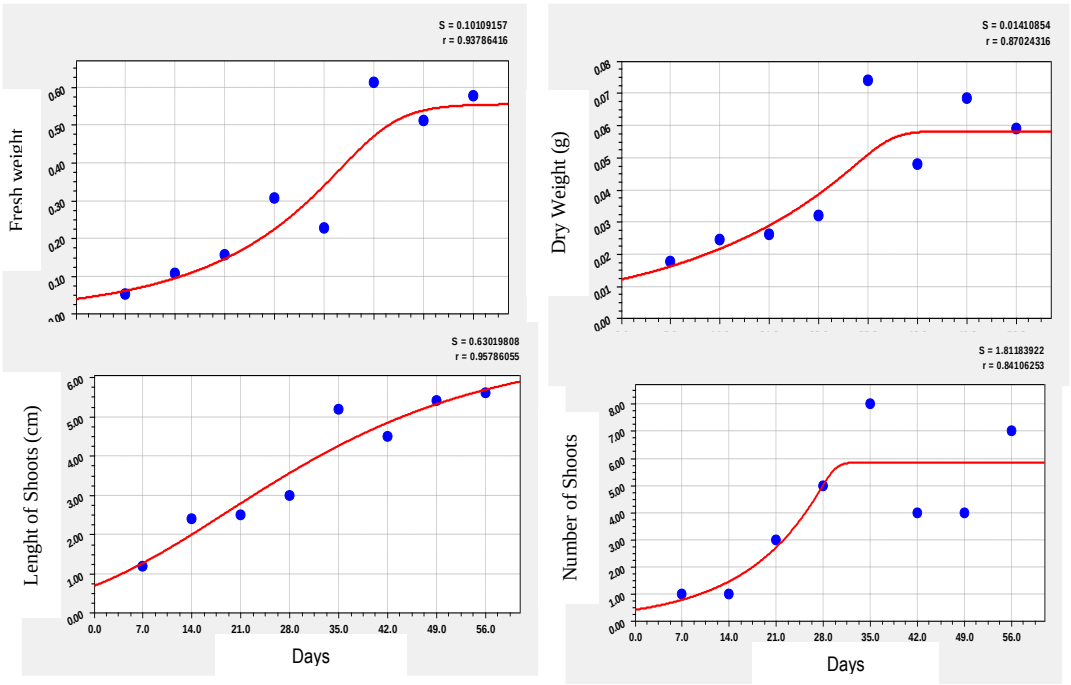

Fig 1: Growth curve of axillary shoots for 56 days culture, based on (a) fresh weight, (b) dry weight, (c) shoot length, and (d) number of shoots.

Axillary shoot growth was influenced by sucrose concentration. It was showed by number of shoots and shoots length data (Table 1 and Fig. 2A, D, G, J, M). Low concentration of sucrose (10 g/L) caused limited growth of shoot, even in combination treatment with erythrosa 4-P or without erythrosa 4-P. The best treatment to induced shoot growth was supplementation of sucrose 50 g/L and erythrose 4-P 2.5 µM. Based on number of leaves data, supplementation of sucrose 10 g/L without erythrose 4-P and combination with 2.5 µM erythrose 4-P have lowest number of leaves than other treatments. In this research supplementation of erythrose 4-P could not influenced growth of axillary shoot, which was showed by number of shoots, shoots length and number of leaves data. Therefore, it could be concluded growth of axillary shoot of *G. procumbens* was only influenced by sucrose concentration. The best sucrose concentration to increased number of shoots was 30 g/L, shoots length was 50 g/L and number of leaves was 30 g/L. In case of number of shoots, this results almost same as Keng *et al.;* [14] results. They obtain mean of number of shoots 8.9 - 18.2, therefore means of shoots was 15.2 in this research.

Table 1: Effect combination of sucrose and erythrose 4-P on growth of axillary shoots of *Gynura procumbens*

Treatments		Fresh weight (g)	Dry weight (g)	Number of shoots	Shoots length (cm)	Number of leaves
Sucrose (g/L)	Erythrose 4-P (µM)					
10	0	0.32±0.01^{ab}	0.01±0.00^{a}	6.2±0.84^{b}	3.12±0.29^{ab}	16.2±1.09^{a}
30	0	1.61±0.88^{g}	0.07±0.05^{b}	15.2±6.9^{c}	5.34±1.05^{d}	49.8±15.0^{de}
50	0	0.72±0.23^{bcde}	0.11±0.12^{b}	3.8±2.77^{a}	3.67±0.15^{a}	30.6±8.96^{ab}
10	1	0.54±0.07^{abcd}	0.03±0.00^{a}	7.4±4.61^{b}	2.78±1.32^{a}	33.0±12.5^{abc}
30	1	0.84±0.49^{cde}	0.17±0.30^{b}	7.8±3.03^{b}	4.72±0.96^{bc}	31.0±5.33^{abc}
50	1	1.12±0.40^{ef}	0.17±0.30^{b}	5.2±0.84^{a}	6.16±1.99^{de}	21.2±4.20^{abc}
10	2.5	0.17±0.03^{a}	0.06±0.04^{b}	5.2±3.35^{a}	2.34±0.72^{a}	17.6±4.87^{ab}
30	2.5	0.84±0.43^{cde}	0.06±0.04^{b}	9.6±4.28^{b}	5.14±0.93^{cd}	35.4±15.3^{c}
50	2.5	1.69±0.38^{g}	0.14±0.03^{b}	6.0±3.08^{b}	7.72±2.10^{e}	22.0±7.77^{abc}
10	5	0.49±0.08^{abcd}	0.02±0.00^{a}	9.2±2.86^{bc}	3.26±0.24^{abc}	26.8±5.16^{abc}
30	5	1.41±0.12f^{g}	0.25±0.39^{b}	11.2±2.86^{c}	5.78±0.85^{de}	53.8±12.2^{e}
50	5	1.79±0.42^{g}	0.12±0.02^{b}	3.8±2.86^{a}	6.35±1.85^{cd}	28.7±6.23^{bc}
10	25	0.39±0.06^{abc}	0.02±0.00^{a}	12.6±1.52^{c}	2.42±0.58^{a}	31.0±6.51^{abc}
30	25	0.93±0.09^{de}	0.05±0.01^{a}	6.6±1.52^{ab}	4.82±0.14^{bc}	32.8±4.76^{abc}
50	25	0.54±0.06^{abcd}	0.03±0.00^{a}	8.4±1.52^{bc}	3.30±0.48^{abc}	36.6±7.50^{cd}

Data followed by same letter indicate no significant different at α=0, 05 based on Gomes-Howel test. Morphology of the axillary shoot in many treatments was showed in Figure 2. Axillary shoot growth in medium with supplemented sucrose 10 g/L lower than 30 and 50 g/L, whereas height of axillary shoot in medium with supplemented sucrose 50 g/L higher than 30 g/L, but number of axillary shoot was lower. The highest dry weight was gotten in combination treatment of sucrose 30 g/L and erythrose 4-P 5 µM (0.25 g). This data was also support with mean number of leaves (Table 1, Figure 2K).

Effect of sucrose and erythrose 4-P on flavonoid production

Analysis of flavonoid production was done by qualitative and quantitative methods. Result showed that all of samples have violet spot on TLC silica gel 60 F_{254} (Merck) under UV light at 366 nm (data not showed). Results of quantitative analysis of flavonoid compound in axillary shoot of *G. procumbens* were showed in Table 2.

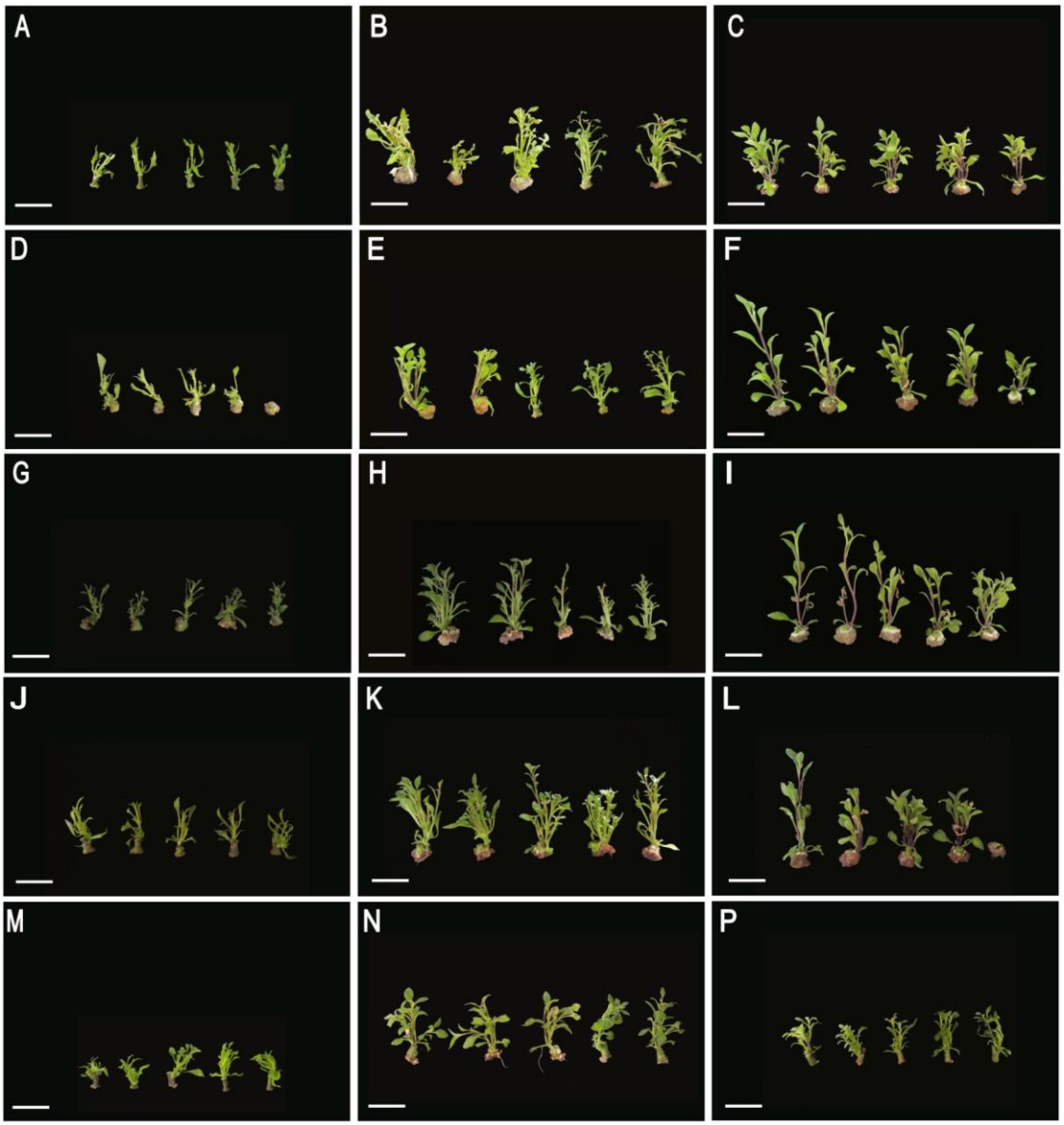

Fig 2: Axillary shoots of Gynura procumbens after 42 days culture in various concentration of sucrose and erythrose 4-P. (A) S10E0; (B) S30E0; (C) S50E0; (D) S10E1; (E) S30E1; (F) S50E1; (G) S10E2,5; (H) S30E2,5; (I) S50E2,5; (J) S10E5, (K) S30E5, (L) S50E5, (M) S10E25, (N) S30E25, (P) S50E25. S: sucrose, E: erythrose 4-P, bar = 3 cm

Table 2: Flavonoid content of ethanol extract of axillary shoots in various combination of sucrose and erythrose 4-P

No.	Treatments		Cathecine	Quercetin	Kaempferol
	Sucrose (g/L)	Erythrose 4-P (µM)	(µg/mg dry weight)		
1.	10	0	0.24	0.926	$0.423x10^{-6}$
2.	30	0	0.64	0.754	$0.321x10^{-6}$
3.	50	0	0.66	1.395	$0.466x10^{-6}$
4.	10	1	0.30	0.445	$0.149x10^{-6}$
5.	30	1	0.48	2.092	$0.811x10^{-6}$
6.	50	1	0.66	1.161	$0.436x10^{-6}$
7.	10	2.5	0.34	0.581	$0.349x10^{-6}$
8.	30	2.5	0.54	1.286	$0.502x10^{-6}$
9.	50	2.5	0.56	0.966	$0.408x10^{-6}$
10.	10	5	0.34	0.798	$0.266x10^{-6}$
11.	30	5	0.18	0.991	$0.399x10^{-6}$
12.	50	5	0.22	0.798	$0.318x10^{-6}$
13.	10	25	0.16	1.402	$0.467x10^{-6}$
14.	30	25	0.26	1.714	$0.694x10^{-6}$
15.	50	25	0.56	0.979	$0.555x10^{-6}$
16.	Ex vitro axillary shoots		0.26	1.590	$0.668x10^{-6}$

High content of cathecine was found in two treatment, there are supplemented of sucrose 50 g/L without erythrose 4-P and combination of sucrose 50 g/L and erythrose 4-P 1 µM. Data also showed that increasing supplementation of sucrose will increasing cathecine content in all treatment, so cathecine content was influenced by sucrose concentration. The higher concentration of sucrose, the higher content of cathecine, although supplementation of erythrose 4-P in low concentration. Most of treatments could produce higher cathecine than ex vitro axillary shoots. Data also showed that increasing of erythrose 4-P resulted low of cathecine content (Table 2). Production of quercetin and kaempferol in all treatment had same trends. It could be caused by both of its compounds had more similar structure. If we investigated the trends of data, we knew that in low sucrose treatments (10 g/L) content of quercetin and kaempferol was low (it was showed at treatments no. 4,7,10, and 13), in normal sucrose treatments (30 g/L) content of quercetin and kaempferol was higher than high sucrose treatments (50 g/L) which was showed at treatments no. 5, 8, 11, and 14. Supplementation of erythrose 4-P could increase production of quercetin and kaempferol, if it was added normal sucrose concentration, although in most of treatments content of quercetin and kaempferol almost same as ex vitro axillary shoots. The highest concentration of quercetin and kaempferol was achieved in medium supplemented with sucrose 30 mg/L and erythrose 4-P 1 µM.

Concentration of sucrose 30 g/L was optimum in medium without erythrosa 4P. It was showed by number of shoots, shoots length, and number of leaves. Result of this experiment have the same trend with Yoon et al. [14] research, which produced the highest biomass of *Anoectochilus formosanus* in MS medium supplemented with sucrose 30 g/L. Supplementation of sucrose 60 and 90 g/L caused decreasing of biomass. In this research biomass of axillary shoots also decreased in medium that was supplemented sucrose 50 g /L. High concentration of sucrose (more than 10 g/L) could influence biomass production of axillary shoots of *G. procumbens.* The same result was showed in cell growth of *Melastoma malabathricum* [16] and cell culture of strawberry [17].

Decreasing of cell growth correlated with inhibition absorption of mineral nutrition that was caused by highly osmotic pressure. In the highly osmotic pressure plant cell could not absorb mineral nutrition, but only absorb water, so the fresh weight became highly than the lower osmotic pressure. It's showed plant cell contain highly water, so the cell have the highest fresh weight and the lowest dry weight. In this experiment, supplemented medium with sucrose 30 g/L have the highest dry weight biomass. It's showed that sucrose 30 g/L was the best concentration to axillary shoot growth and it's same with the other plant that was grown in tissue culture. Sucrose was used as carbon source for plant growth and source of energy. Besides that, sucrose also necessary carbon source for production of secondary metabolite.

Supplementation of erythrose 4-P in low concentration could increase biomass production. Erythrose 4-P was intermediate compound that was produced in general biosynthesis of secondary metabolite. Erytrhose-4P was produced from sucrose. Sucrose was reduced to glucose and glucose-6P. Glucose-6P was reduced to two compounds; there were phosphoenol pyruvic acid that continues formed primer

metabolite and erythrose 4-P which have function as precursor of secondary metabolite. How the erythrose 4-P influenced growth of plant biomass cans has not known yet, but in another research found that supplemented of precursor, such as supplemented of phenylalanine could improve biomass production in *Ocimum sanctum* cell cultures [18].

When supplementation erythrose 4-P was followed by higher sucrose, production of cathecine higher than combination of erythrose 4-P and low concentration of sucrose. It's showed that supplementation of sucrose in high concentration could induced production of secondary metabolite. Many research showed that gene code of enzyme that involved producing phenyl propanoid, could influenced by carbohydrate dissolved, such as glucose and sucrose that was used as signal molecule [19]. Flavonoid was group of phenolic compound. According to Ghasem zadeh and Ghasem zadeh [9], production of phenolic compound was also catalyzed by phenylalanine ammonia lyase (PAL). Erythrose 4-P was intermediate compound of secondary metabolite biosynthesis and was synthesis from sucrose.

CONCLUSION

The highest biomass (dry weight) was achieved in medium supplemented with high concentration of sucrose and erythrose-4-phosphate, whereas high content of flavonoid (cathecine, quercetine, and kaempferol) were achieved in medium supplemented with high sucrose and low concentration of erythrose 4-P. Sucrose concentration has significant effect to influenced flavonoid production than erythrose 4-P. Biomass production of Axilary shoot of *G. procumbens* have no positive correlation with flavonoid production, so supplemented of sucrose and erythrose 4-P would be adapted to the needs, whether to produce biomass or flavonoid.

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