Effect of sucrose, erythrose-4-phosphate and phenylalanine on biomassa and flavonoid content of callus culture from leaves of Gynura procumbens Merr.

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Effect of Sucrose, Erythrose-4-Phosphate and Phenylalanine on Biomassa and Flavonoid Content of Callus Culture from Leaves of *Gynura procumbens* Merr.

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Abstract. The aims of this study were to know the effect of concentration of sucrose, erythrose-4-phosphate and phenylalanine on biomass and flavonoid content of callus cultures from leaves of sambung nyawa (*Gynura procumbens* Merr.). This study was experimental research with complete randomized design. Callus induction was treated in MS medium supplemented with NAA 2 mg/L, BAP 1 mg/L and sucrose concentration (10 g/L, 30 g/L and 50 g/L) respectively were combined with erythrose-4-phosphate (0 µM, 2,5 µM and 5 µM) and phenylalanine (0 mg/L, 2 mg/L and 3 mg/L), each treatment were repeated four times. After six weeks of culture, fresh and dry weight of calli were measured and extracted with ethanol absolut. Crude extract ethanolic of callus was analyzed used by a modified colorimetric with spectrophotometer method. The best yield of calli biomass (0,672 ± 0,112 gram of fresh weight and 0,033 ± 0,009 gram of dry weight) was obtained in treatment of 30 g/L sucrose of and 5 µM erythrose-4-phosphate. The highest total flavonoid content was obtained of calli treated with 30 g/L of sucrose and 3 mg/L of phenylalanine (3633,4 ppm quercetin/gram dry weight and 15777,8 ppm kaempferol/gram dry weight).

INTRODUCTION

G. procumbens are known as traditional medicine plants in Indonesia, Malaysia, Thailand and other South East Asian countries [1]. People use *G. procumbens* leaves as a cure for diabetes, hypertension, urinary tract infection, and could also be used as anti-inflammatories and anti-allergies [2]. Based on the research conducted by Rosidah *et al.* [3], *G. procumbens* leaf extracts contain a flavonoid type compound, which is kaempferol-3-O-rutinoside and astragalin. The flavonoid compounds act as anti-allergy, antivirus, antitumor, anti-oxidants, anti-inflammations [4], anticancer [5] and anti-bacterias [6].

The use of *G. procumbens* plants *in vitro* is to obtain flavonoid compound, which has not much been implemented. The agriculture *in vitro* towards these plants are related with the effort of increasing the sprouts using stem nodes as inducted explants, forming axillary branches in MS-media (Murashige dan Skoog) with the addition of BA (benzyladenine) and NAA (naphtalene acetic acid) growth substance regulator [2]. However, the use of *G. procumbens* leafs as callus culture explants in order to obtain a secondary metabolite compound have not yet been researched.

The increase of flavonoid compounds in these plants have relations with the variety of precursors and compounds towards their biosynthesis. Flavonoid biosynthesis can be undertaken through two pathways, one is the shikimate pathway, and the other is the malonate acid pathway. Both of these biosynthesis play the role in determining the carbon flavonoid framework [7].

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The sikhimate pathway is the biosynthesis pathway for a variety of secondary metabollite compounds other than flavonoid, such as phenolates, lignins, lignans and stilbenes [8]. The primary stage in sikhimate pathway is condensing erythrose-4-phosphate obtained from pentose phosphate pathway, where subsequently it will form three aromatic amino acids, which are tryptophan, tyrosine and phenylalanine. Based on these facts, the erythrose-4-phosphate is a precursor for the sikhimate pathway which will produce flavonoid [9]. In this research, erythrose-4-phosphate was used as a precursor in order to increase the flavonoid on callus. Besides, the phenylalanine was also an aromatic amino acid, where in this research, it was used to increase the callus flavonoid level from *G. procumbens* Merr. Phenylalanines are mostly used in the effort to increase the flavonoid *in vitro*, such as in the research on *Hydrocotyle bonariensis* callus culture [10].

Sucrose is the main source of carbon on culture media which, besides from functioning to induct the cell growth so that the callus biomass increases, it also helps the molecule signals to stimulate the genetic expressions in coding the enzymes involved in isoflavonoid biosynthesis [11]. This research is aimed to investigate the effects of varied sucrose erythrose-4-phosphate and phenylalanine concentrations on production of biomass and falavonoid content of callus culture of *G. procumbens* from leaf explants.

METHODS

Preparing The Media

The culture media was MS-media (Murashige and Skoog) which contained 0,8% of agar, 2 mg/L of NAA and 1 mg/L of BAP. The amount of sucrose concentration variation added to the media was 10 g/L, 30 g/L and 50 g/L. Furthemore, the media treatment being applied was by combining each of the sucrose concentrations (10 g/L, 30 g/L and 50 g/L) with concentrations of erythrose-4-phosphate (0 µM, 2,5 µM dan 5 µM) and other media, which was done by combining the sucrose and phenylalanine (0 mg/L, 2 mg/L dan 3 mg/L). Adding erythrose-4-phosphate and phenylalanine onto the media was done inside the Laminar Air Flow (LAF) because the media would not be able to withstand the high temperature given by the autoclave.

Callus Culture

G. procumbens Merr. were obtained from Kayon Market and cultivated independently by stem cuttings. The leaf used was the third, fourth and fifth leaf counted from the top end of the stem. Just before the cultivation process is started, the leaves are picked and washed with detergents and subsequently rinsed three times. Inside the LAF, the leafs are sterilized using 20% clorox (v/v) for 7 minutes and rinsed 3 times, with sterilized demineralized water. The leaves are drained on a filter paper and cut right in the middle where the leaf bone is, therefore obtaining leaf explants with an area of ± 1 cm^2. The leaf explants are then ready to be planted on the prepared MS-media. Leaf explants were cultivated in incubation room at 25 ± 3ºC with white neon lighting. After 6 weeks of cultivation period, *G. procumbens* leaves are then ready to be harvested.

Harvesting was done by separating the callus from the explants and weighing the fresh weight of the callus using an analytic scale. Next, the callus are then wrapped with aluminium foil and stored in an oven at 60 ºC until the callus are dried and have a constant mass.

Determining the Total Flavonoid Contents

Dried callus are weighed (0.05 g) and crushed using mortar until they become a fine powder. Next, the fine powder was extracted using a 5 mL of absolute ethanol. The extraction process was done under a water bath at 60^{o}C for 5 minutes in which then it is filtered with filter paper. The extract was concentrated until it reached a volume of 2 mL. Extractions were also done on dried *G. procumbens* leaves using the same method as the callus extraction. This was carried out in order to compare the compounds contained in the plants and callus.

Before obtaining the total flavonoid contents from total callus samples, the quercetin and kaempferol curves have to be determined beforehand. The total flavonoid contents were measured using modified colorimetry method [12]. The callus ethanol extracts from each varied concentration treatments of sucrose, erythrose-4-phosphate and phenylalanine were each taken by 0,25 mL, added into 1,25 mL of demineralized water and 75 µL of 5% sodium

nitrate solution, and dissolved for 6 minutes. Then, 0,5 mL of NaOH 1 M and demineralized water were added into the solution until it reached a volume of 2,5 mL. The absorbance value was obtained using UV-Vis spectrophotometer at 510 nm wavelength. The blanks used were absolute ethanol. The absorbance values obtained were counted using linear regression equations based on the standard quercetin and kaempferol curves, therefore obtaining the total flavonoid contents from the samples.

Statistic Analysis

Each treatments were repeated four times. The obtained callus fresh and dry weights significance were retrieved based on the multivariate analysis using SPSS 17 software. On the other hand, in order to gain the significant difference from each of the treatments, Duncan Test was used. The total flavonoid contents were analyzed descriptively.

RESULTS AND DISCUSSION

Effects of Sucrose and Erythrose-4-Phosphate on Callus Biomass

Treatment of combined 30 g/L sucrose and 5 µM erythrose-4-phosphate gave an highest average of fresh and dry weights of each 0,672 grams and 0,033 grams respectively. And on the other hand, the lowest both fresh and dry weights resulted from the treatment of combined 10 g/L sucrose and 5 µM erythrose-4-phosphate, with each 0,286 grams and 0,013 grams respectively (see Figure 1).

In this research, the right concentration combination of sucrose and erythrose-4-phosphate was capable of increasing the callus biomass. Combinations of 30 g/L of sucrose and 2,5 µM of erythrose-4-phosphate, and 30 g/L of sucrose and 5 µM of erythrose-4-phosphate, both were able to yield in higher biomass compared to 30 g/L of sucrose without an addition erythrose-4-phosphate. Meanwhile, 50 g/L of sucrose combined with erythrose-4-phosphate was precisely adequate of producing lower biomass compared to a single 50 g/L of sucrose. This suggests that a 30 g/L concentration of sucrose was the right amount of concentration to be mixed with erythrose-4-phosphate in comparison to a 50 g/L sucrose concentration.

Direct interactions of the phenanthroline and the manganese salt in solution produced a yellowish cationic complex which could be precipitated on the addition of triflate anions. The electrical equivalent conductance of this complex was recorded with respect to the known ionic simple compounds in aqueous solution, and the result is shown in Table 1. It suggests that the corresponding value is in the range of ionic compounds with three ions per molecule, and thus the possible empirical formula of $[Mn(phen)_n](CF_3SO_3)_2 \cdot xH_2O$ is then proposed for this complex.

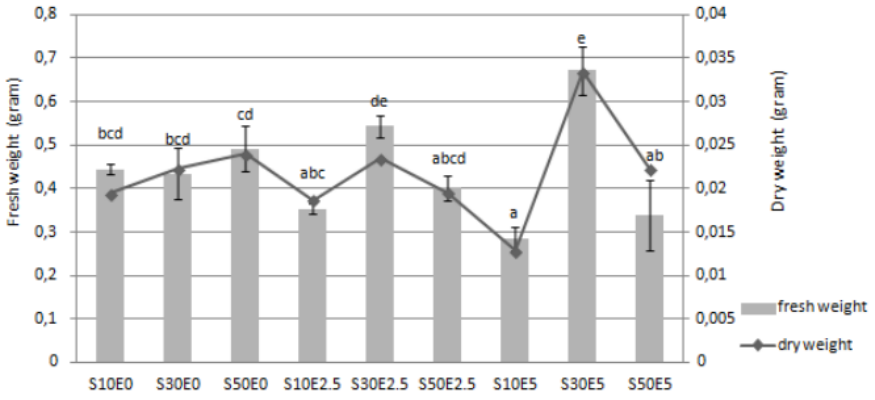

FIGURE 1. The average fresh and dry weights of callus in varied concentration of sucrose and erythrose 4-P. Different letters show that there is a significant difference based on the Duncan Test with $\alpha = 0,05$.

S10E0 : sucrose 10 g/L, erythrose 4-P 0
S30E0 : sucrose 30 g/L, erythrose 4-P 0
S50E0 : sucrose 50 g/L, erythrose 4-P 0
S10E2.5 : sucrose 10 g/L, erythrose 4-P 2.5 µM
S30E2.5 : sucrose 30 g/L, erythrose 4-P 2.5 µM
S50E2.5 : sucrose 50 g/L, erythrose 4-P 2.5 µM
S10E5 : sucrose 10 g/L, erythrose 4-P 5 µM
S30E5 : sucrose 30 g/L, erythrose 4-P 5 µM
S50E5 : sucrose 50 g/L, erythrose 4-P 5 µM

In this research, efforts of increasing the biomass and flavonoid contents in *G. procumbens* callus using a combined concentrations of sucrose and erythrose-4-phosphate and sucrose and phenylalanine have been executed. The effects of erythrose-4-phosphate mechanisms towards callus biomass have not yet been able to be explained. The callus biomass increase is obviously started with the process of dividing the cells. Cells splitting was also implemented during the process of plant buds cultivation. The research carried out by Beaudoin-Eagan dan Thorpe [14] demonstrated that there were activities of sikhimate pathways that were involved during the whole bud initiation in tobacco callus cultivation (*Nicotiana tabacum* L.).

Throughout the bud initiation process in tobacco callus, it was found that there were enzymes that had crucial roles, which are kinase sikhimate, chorismate mutase, and anthranilic kinase [14]. These enyzmes are produced from sikhimate pathway which needed erythrose-4-phosphate as precursors [15]. Sikhimate kinase is an enzyme that contributes to producing ATP needed by plant cells as metabolism energy used for cell division and growing. By doing so, erythrose-4-phosphate also have an impact in forming and increasing the callus cells mass. This research have verified this analysis, and it is shown by adding 5 µM of erythrose 4-P, which emerged in the highest callus biomass.

Effects of Sucrose and Phenylalanine on Callus Biomass

Based on observations, the best average fresh and dry weights were derived from a concentration of 50 g/L of sucrose and 3 mg/L of phenylalanine, where each have a weight of 0,527 grams and 0,026 grams respectively. And at the same time, the lowest average was given by concentration treatment of 30 g/L of sucrose combined with 2 mg/L of phenylalanine, with combined fresh and dry weight of 0,147 grams and 0,010 grams respectively (see Figure 2).

Out of all the sucrose and phenylalanine combination treatments, the highest callus biomass was gathered from a combination treatment of 50 g/L of sucrose with 3 mg/L of phenylalanine. This result was higher compared to the treatment of sucrose without adding phenylalanine. It was proven that the right amount of sucrose and phenylalanine concentration was able to increase the callus biomass. Due to this fact, the increase of callus biomass, besides from being affected by the amount of sucrose, it was also altered by the given presence of phenylalanine. However, a combined concentration of 30 g/L of sucrose and 5 µM of erythrose-4-phosphate proceeded in the best callus biomass out of all the treatments. The addition of phenylalanine slowed down the callus growth, apart from the treatment of adding 50 g/L of sucrose and 3 mg/L of phenylalanine (shown on Figure 2).

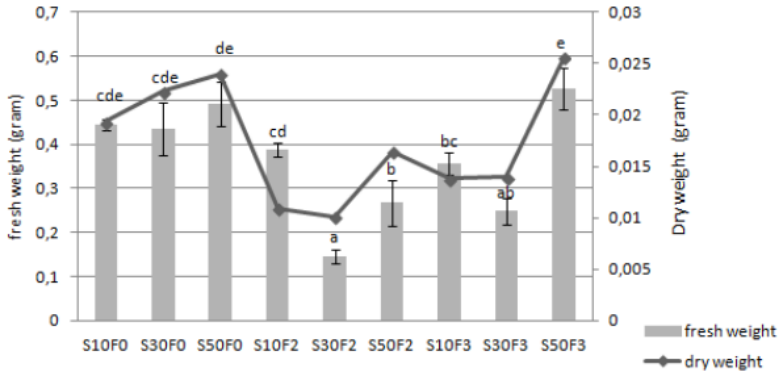

FIGURE 2. The average fresh and dry weights of callus in varied combined concentration of sucrose and phenylalanine. Different letters show that there is a significant difference based on the Duncan Test with $\alpha = 0,05$.

S10F0 : sucrose 10 g/L, phenyalanine 0
S30F0 : sucrose 30 g/L, phenyalanine 0
S50F0 : sucrose 50 g/L, phenyalanine 0
S10F2 : sucrose 10 g/L, phenyalanine 2 mg/L
S30F2 : sucrose 30 g/L, phenyalanine 2 mg/L
S50F2 : sucrose 50 g/L, phenyalanine 2 mg/L
S10F3 : sucrose 10 g/L, phenyalanine 3 mg/L
S30F3 : sucrose 30 g/L, phenyalanine 3 mg/L
S50F3 : sucrose 50 g/L, phenyalanine 3 mg/L

Adding phenylalanine gave a better outcome compared to erythrose-4-phosphate in increasing the callus biomass. This can be interpreted through a couple of studies on the roles of phenylalanine in phytohormones biosynthesis. Phenylalanine together with ornithine is an amino acid that benefited on gibberellin biosynthesis, which is a plant hormone that have impacts on the growth and development of a plant [16]. Gibberellin is a hormone that has a crucial role in cell growth. The effects of gibberellin in increasing the stem elongation have been known since 1930 through the discovery of pathogen fungi *Gibberella fujikuroi*, where during this time, gibberellic acid caused uncontrollable stem elongation on rice [8]. Due to this finding, synthesized gibberellin which was carried out due the presence of phenylalanine caused so much uncontrollable callus cells growth that the cells mutated exponentially. The addition of sucrose in the research also benefited in increasing the callus biomass. Many researchers have known that sucrose is a main source of carbon for the plants network under the conditions of *in vitro*, mainly when the plants cells are still not yet able to perform photosynthesis [17]. The availability of sucrose on culture media could increase the cells growth because it can supply all the carbon sources needed in a variety of cell metabolism processes [18].

Effects of Sucrose, Erythrose-4-Phosphate, and Phenylalanine on Flavonoid Content

A sucrose concentration of 30 g/L without adding erythrose-4-phosphate was able to deliver the best total flavonoid content, which was 1966 ppm quercetin/grams of dry weight and 10222,2 ppm kaempferol/grams of fresh weight (Table 1). A 50 g/L of sucrose and 5 µM of erythrose-4-phosphate concentration gave a lower total flavonoid compared to a 10 g/L of sucrose and 2,5 µM of erythrose-4-phosphate concentraion. In this research, it was confirmed that adding erythrose-4-phosphate was not able to increase the flavonoid contents in comparison to the addition of a single sucrose.

The use of erythrose-4-phosphate as a flavonoid biosynthensis precursor on *in vitro* cultivation have not been much done yet. Few researches used elicitors such as methyl jasmonate, on a concentration of 100 M, and was able to present the highest rosmarinic acid on *Ocimum sanctum* cell cultivation [13]. The use of precursors as well as elicitors was basically intended to induce the production increase of secondary metabolite compounds on plant cells through network cultivation techniques.

TABLE 1. The total flavonoid contents on varied combined concentrations of sucrose and erythrose-4-phosphate

Treatments		Total flavonoid content (ppm quercetin/gram of dry weight)	Total flavonoid content (ppm kaempferol/gram of dry weight)
Sucrose (g/L)	Erythrose-4-Phosphate (µM)		
10	0	1433,4	8444,4
30	0	1966,6	10222,2
50	0	700	6000
10	2,5	1833,4	9777,8
30	2,5	900	6666,6
50	2,5	1300	8000
10	5	766,6	6222,2
30	5	1500	8666,6
50	5	900	6666,6
Ex vitro		10100	37333,4

The increase of flavonoid contents was also carried out using a variety of compounds that are capable of inducting flavonoid biosynthesis using phenylalanine aromatic amino acid. Based on Table 2, the highest total flavonoid contents produced by concentration treatment of 30 g/L of sucrose and 3 mg/L of phenylalanine, where both have a combined 3633,4 ppm quercetin/gram of dry weight and 15777,8 ppm kaempferol/gram of dry weight. The produced flavonoid contents using this type of treatment was proven to be twice as much when compared to the concentration treatment of 10 g/L sucrose and 2,5 µM of erythrose-4-phosphate.

Other researches that used phenylalanine as a treatment to increase the secondary metabolite compound production was also implemented by Masoumian *et al.* [10], where the results suggested that 3 mg/L of phenylalanine concentration in a solid MS-media combined with a concentration of 3% sucrose was able to execute the highest *Hydrocotyle bonariensis* callus. Unfortunately, in this research the amount of quercetin and *G. procumbens* callus kaempferol were still lower in comparison to the amount of quercetin and kaempferol inside the leaves (ex vitro).

TABLE 2. The total flavonoid contents on the varied combined concentrations of sucrose and phenylalanine

Treatment		Total flavonoid contents (ppm quercetin/gram of dry weight)	Total flavonoid contents (ppm kaempferol/gram of dry weight)
Sucrose (g/L)	Phenylalanine (mg/L)		
10	0	1433,4	8444,4
30	0	1966,6	10222,2
50	0	700	6000
10	2	2700	12666,6
30	2	366,6	4888,8
50	2	2833,4	1311,2
10	3	1700	9333,4
30	3	3633,4	15777,8
50	3	1500	8666,6
Ex Vitro		10100	37333,4

In terms of flavonoid contents, this research also presented that there were effects given by the combined concentrations of sucrose, erythrose-4-phosphate, and phenylalanine. The use erythrose-4-phosphate as a flavonoid biosynthesis precursor in *in vitro* cultivation have not yet much been done. The option of choosing to implement treatment towards erythrose-4-phosphate is based on the flavonoid biosynthesis concepts which also engaged the

aforementioned compound. Even so, the inclusion of single sucrose was proven as a better method in increasing the flavonoid contents compared to when combined with erythrose-4-phosphate.

Flavonoid was synthesized through the sikhimate pathway which needed erythrose-4-phosphate as a precursor [15]. Erythrose-4-phosphate was formed through primary metabolism which involved sucrose through pentose phosphate pathway [19]. It was predicted that the obtained erythrose-4-phosphate concentration through pentose phosphate pathway had a higher concentration in comparison to the treated erythrose-4-phosphate. This justified that sucrose had more impact towards the increase of total flavonoid contents in comparison to the addition of erythrose-4-phosphate. Sucrose also had effects in activating the enzymes that contributed in flavonoid biosynthesis in sikhimate pathway, and these enzymes are PAL (phenylalanine ammonia-lyase), CHS (chalcone synthase), CHI (chalcone isomerase) dan IFS (isoflavone synthase) [15].

Nonetheless, phenylalanine treatment gave the best reaction in increasing the callus flavonoid contents. The phenylalanine availability on culture media is a key for other enzyme activities due to its role as culture media for other enzyme activities since it contributes as a substrate for PAL enzyme. Adding phenylalanine will shortened the sikhimate pathway in flavonoid synthesis, therefore the end product is able to be manufactured faster and in higher quantity.

CONCLUSION

Based on the research taken, it can be said that concentration treatment of 30 g/L of sucrose added with 5 µM of erythrose-4-phosphate concentration was able to increase the callus biomass in comparison to other treatments. However, applying erythrose-4-phosphate on the other hand decreased the flavonoid contents on callus. This was shown that by treating 30 g/L of sucrose without adding erythrose-4-phosphate was able to produce the highest flavonoid contents. By doing so, adding erythrose-4-phosphate decreased the callus flavonoid contents in *G. procumbens* Merr.

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