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Effects of Polyethylene Glycol (PEG) on Growth and Quercitrin Content of Shoot Cultures of *Chrysanthemum morifolium* Ramat cv. Yulimar

Tia Setiawati[1*], Mohamad Nurzaman[1], Anandira W. Lestari[1], Annisa[1], Valentina K. Adimurti[2] & Ichsan N. Bari[3]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Jalan Raya Bandung Sumedang Km 21, Sumedang, Jawa Barat 45363, Indonesia.
[2] Department of Chemistry, Faculty of Science and Informatics, Universitas Jendral Ahmad Yani
Jalan Terusan Jendral Sudirman, Cimahi, Jawa Barat 40285, Indonesia
[3]Department of Plant Pest and Disease, Faculty of Agriculture, Universitas Padjadjaran. Jalan Raya Bandung Sumedang Km 21, Sumedang, Jawa Barat 45363, Indonesia.

*Corresponding Author
E-mail: tia@unpad.ac.id

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ABSTRACT

Chrysanthemum morifolium consisted of secondary metabolites such as kaempferol, luteolin, quercitrin, quercetin, and morin. The potential alternative method to increase the production of secondary metabolites in vitro is to grow shoots cultures in growth medium supplemented with Polyethylene Glycol (PEG) as an elicitor. The aim of this research was to obtain the best PEG concentration which can produce the highest quercitrin content in chrysanthemum shoots culture. The experiment consisted of the five concentrations of PEG 6000 namely 0 ppm, 10 ppm, 20 ppm, 30 ppm, and 40 ppm added to Murashige and Skoog (MS) media. Data was taken after 45 days after of culture. The parameters observed were the height of shoots, the number of leaves, dry weight of micro-shoots, and quercitrin content analyzed using High Performance Liquid Chromatography (HPLC). The result showed that 10 ppm PEG concentration addition produce the highest shoots height, dry weight of micro-shoots and content of quercitrin were 8.99 cm; 0.1373 grams; and 0.02749 mg/g DW respectively. The growth of Chrysanthemum was directly proportional to quercitrin content. Quercitrin content in Chrysanthemum shoots cultures decreases with increasing PEG concentrationin medium.

Keywords: *Chrysanthemum morifolium*, growth, polyethylene glicol, quercitrin

Introduction

Chrysanthemum morifolium has been widely used in traditional Chinese herbs as herbal tea or medicine for thousands of years [1]. Japan uses *Chrysanthemum* as an a medicine for eyes and it improves the vision system [2].

The results of phytochemical screening in *C. morifolium* showed that there were 12 types of flavonoids and 58 volatile compounds [3]. These flavonoids include kaempferide, baicalein, kaempferol, luteolin, quercetin, morin, myricetin, baicalin, hyperoside, luteoloside, myricitrin, and buddleoside. Types of flavonoids that are found in *C. morifolium* are mostly quercetin-3-O-rhamnoside (quercitrin) and luteolin-7-glucoside, which are derivative compounds of quercetin [4, 3, 5]. Quercitrin is a bioflavonoid with antioxidant properties that has high absorption compared to other quercetin forms. Quercitrin has antimicrobial, antitumor, anti-allergic effects, and can protect the skin from UV light [6].

Tissue culture or *in vitro* techniques are potential alternative methods for increasing secondary metabolite production due to several advantages. *In vitro* techniques can effectively produce secondary metabolites [7], providing sustainable and renewable medicinal plant resources to produce large-scale secondary metabolites [8]. Several studies reported that shoot culture has the potential to be used for the production of secondary metabolites such as bacoside A in *Bacopa monnieri* [9], baicalin, wogonoside, luteolin, luteolin-7-O-glucoside and verbacoside in *Scutellaria alpine* [10], iridoids, phenolics, flavonoids, and tannins in *Aloe arborescens* [11]. The levels of secondary metabolites produced *in vitro* are often still relatively low [12]. The addition of elicitor is one of the methods to increase the production of secondary metabolites. An elicitor is a biological or non-biological stimulus that can induce synthesis of specific compounds and has an important role in plant adaptation to stress, when added to plant culture [13]. According to [14], the addition of elicitor creates stress conditions, so plants automatically increase secondary metabolites as a form of defense.

Polyethylene Glycol (PEG) is one of the physical elicitors that can induce enzymatic activity and secondary metabolites [15]. Polyethylene Glycol has a high molecular weight, and acts as an osmotic agent that can cause stress on plants. The addition of PEG will reduce the water potential of the culture media, so that explant growth is inhibited and increases the content of secondary metabolites [16]. According to [17], there is a relationship between growth inhibition and increased secondary metabolite production. If a plant is under stress, it will naturally defend itself by producing secondary metabolites and ignoring the growth of other tissues. Increased *in vitro* secondary metabolite production with PEG elicitor has been reported to be successful in several plants, including flavonoid production in cell suspension culture of *Glycyrrhiza inflata* Batal [18], *Hypericum polyanthemum* in the production of uliginosin B [19], silymarin compounds in callus culture of *Silybum marianum* L. [20], as well as vincristine and vinblastine alkaloids in *Catharanthus roseus* callus [21].

The purpose of this study was to determine the effect of PEG on enhancement of quercitrin content of *Chrysanthemum morifolium* Ramat shoot culture.

Materials and Methods

Media preparation and sterilization

The materials used in this study were solidified powder, alcohol, sterile distilled water, acetic acid, 6-benzylaminopurin, Murashige and Skoog media, methanol, acetonitrile, PEG 6000, quercitrin, and sucrose, *in vitro* plantlets of *C. morifolium* Ramat cv. Yulimar.

The PEG concentration treatment consisted of five levels, namely 6000 i.e. 0 ppm, 10 ppm, 20 ppm, 30 ppm, and 40 ppm which was added to MS media + 1 ppm BAP (6-Benzilaminopurin) [22]. Each treatment was repeated five times. Observations were made at 45 days after planting. The parameters observed were shoot height, leaf number, and micro-shoots dry weight, and quercitrin content analyzed using HPLC (High Performance Liquid Chromatography). The study was conducted using an experimental method with a completely randomized design (CRD) and each treatment level was replicated five times. Data were analyzed using Analysis of Variance (ANOVA) and if significantly affected continued by Duncan's Multiple Range Test (DMRT), confidence level 95% ($\alpha = 0.05$), to test the relationship of growth with quercitrin content used Pearson Correlation Analysis.

All equipment and medium to be used were sterilized using autoclaves at a temperature of 121 ° C with a pressure of 15 psi (pounds per square inch) for 15 minutes. The medium used in this study was MS media with the addition of 1 ppm BA as the optimum medium for *Chrysanthemum* shoot culture [23] and PEG in various concentrations of 0, 10, 20, 30 and 40 ppm as treatments. Laminar air flow cabinet before use was sterilized with ultraviolet light and drained with sterile air for 30 minutes, then cleaned with cotton which has been moistened with alcohol.

Explant inoculation

The explants used were *in vitro* single nodes (± 1 cm) with one leaf taken from *C. morifolium* Ramat cv. Yulimar plantlets. Furthermore, explants were planted into the treatment medium, each consisting of four explants. The cultures were incubated at a temperature of 18°C – 25°C with a light intensity of 2000 lux for 45 days. The parameters taken after the end of the experiments.

Quercitrin content analysis

Dry material extraction

The dried and mashed *Chrysanthemum* microshoots were taken as much as 0.25 g then extracted with 10 mL of solution (methanol-acetic acid-aquades,100: 2: 100) at 100 rpm for 1 hour. Then two mL of extract was centrifuged for 10 minutes at 2000 rpm. The solution was then filtered with a cellulose filter membrane (0.22 µm) and the filtrate was used for HPLC analysis [24].

Qualitative and quantiative analysis

The HPLC column types used were C18 (4.6 mm x 250) as the stationary phase and a mixture of methanol: acetonitrile: aquades (10:10:75) containing 5% acetic acid as the mobile phase A and methanol as the mobile phase B. Flow velocity was 1 mL / minute. The UV wavelength used was 125 nm. Qualitative analysis was carried out by comparing retention times between standard quercitrin and sample retention times. Quantitative analysis was done by converting the sample area to a standard area that has known concentrations on a standard calibration curve. The standard calibration curve was obtained from data area of several standard quercitrin concentrations (50, 100, 150 and 200 ppm).

Data analysis

Data were analyzed using Analysis of variance (ANOVA) and if it was significantly followed by Duncan's Multiple Range Test (DMRT) with the confidence level was 95% ($\alpha = 0.05$). Pearson Correlation Analysis was used to test the relationship of growth with quercitrin content of shoot culture.

Results and Discussion

Effect of PEG on Growth of Chrysanthemum

Shoot height

ANOVA results showed that PEG levels in the media has affected shoot height after 45 days of planting (Table 1). The 10 ppm PEG treatment produce the highest average shoot height was 8.99 cm which was significantly different from the others. The lowest average shoot height (6.12 cm) was found in the control (without PEG). Meanwhile, shoot height decreased with increasing PEG concentration up to 40 ppm (Table 1). Similar to the study of [25], as higher concentrations of PEG, resulted in a decline in growth of *Solanum melongena* L. stem. The research of [26] suggested that an increase in the dose of PEG 50-150 gram / L in *in vitro* media resulted in inhibition of growth and regeneration of of *Lathyrus* shoots which was marked by a decrease in the height. According to [27] and [28], an increase in the concentration of PEG resulted in the inhibition of water and nutrient absorption processes caused by a decrease in water potential in the media (low turgor pressure), thereby reducing cell division and cell elongation. Furthermore, increasing PEG concentration in the medium causes a gradual decrease in growth and water content of calluses. High concentrations of PEG caused callus necrosis as well [20].

Table 1. Average height of *C. morifolium* Ramat shoots at various PEG concentrations

Concentrations of PEG	Average shoot height (cm)
0 ppm PEG (control)	6.12 b
10 ppm PEG	8.99 a
20 ppm PEG	7.37 b
30 ppm PEG	7.30 b
40 ppm PEG	6.63 b

Note: The mean value followed by the same letters showed no significant difference ($P < 0.05$).

Figure 1 showed the morphological differences in *Chrysanthemum* micro-shoots treated with PEG in several concentrations with controls. In all PEG treatments, the underside of the stems turns brown and some leaves at the bottom of the stems are withered. Discoloration at the bottom of the shoots becomes brownish and withered leaves due to decreased chlorophyll content in plants. According to [26], drought stress causes oxidative stress in plant cells, so that it can affect the decrease in chlorophyll and carotenoid content.

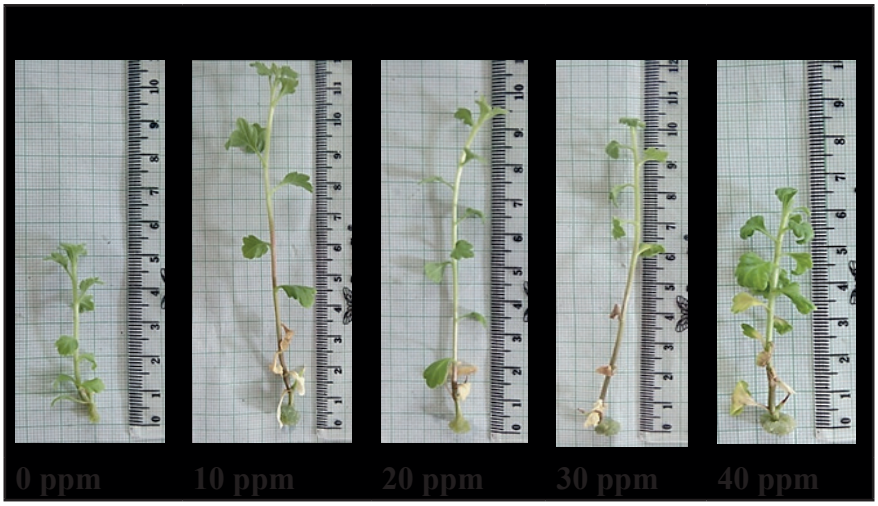

Figure 1. Microshoots of *C.morifolium* Ramat at 45 days after planting with PEG treatment

Number of New Shoots and Leaves

In this study no new shoots were formed so that there was no change in the number of shoots until the end of the observation i.e. one shoot remained. The addition of PEG did not significantly affect the number of leaves based on the results of ANOVA. The results of observations on the number of leaves can be seen in Figure 2.

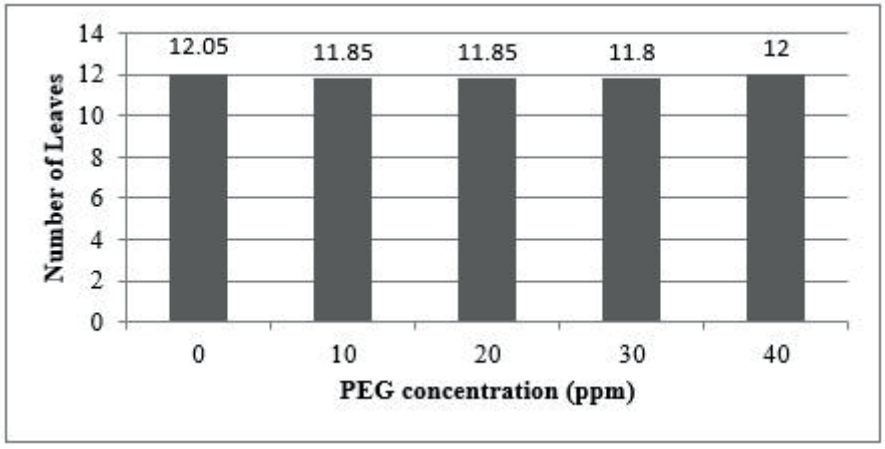

Figure 2. Average number of *C. morifolium* Ramat leaves on various PEG concentration

Dry weight

PEG did not significantly affect dry weight based on ANOVA results. In Figure 3 it can be seen that there was an increase in plantlet dry weight of 5.55% - 26.85% compared to the control. The highest average plantlet dry weight was found in the 10 ppm PEG treatment i.e. 0.137 grams, while the lowest dry weight in the control was 0.108 grams. Similar results occur in eight *Lathyrus* shoot genotypes, as adding 50 - 150 g / L PEG can increase dry weight when compared to controls. Drought stress caused by giving PEG at certain concentrations can increase plantlet dry weight compared to controls [26]. This can be caused by PEG can increase the total dissolved sugar in addition to reducing the amount of water content in plants, so that the plant dry weight of PEG treatment is greater than the control [29]. Soni *et al.* [30] reported that the addition of PEG can increase the accumulation of dry weight of peanut shoots. Increasing the accumulation of dry biomass is related to the two processes of plant productivity, namely dehydration and synthesis of new materials needed for the maintenance of higher osmoticum and continuing it for water absorption.

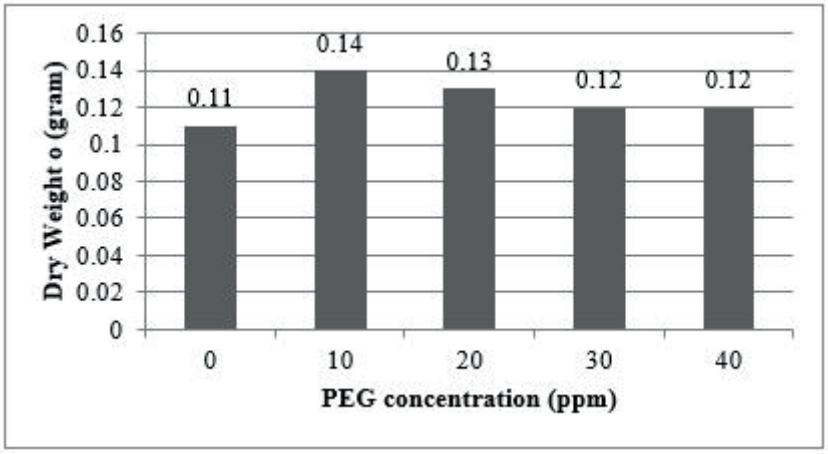

Figure 3. Average dry weight of *C. morifolium* Ramat microshoots on various PEG concentration

Effect of PEG on Quercitrin Content

The ANOVA results showed that using PEG resulted in a significant increase in the content of quercitrin of *Chrysanthemum* shoots. The Duncan's Multiple Range Test results can be seen in Table 2. *Chrysanthemum* shoots at the 10 ppm PEG treatment had the highest quercitrin level of 0.0275 mg / g DW with an increase reaching 344.82%. The lowest lowest quercitrin level was in the control (without PEG) which was 0.0062 mg / g DW. These results showed that the concentration of 10 ppm PEG successfully induced the optimum content of quercitrin compound in *Chrysanthemum* shoot culture, although at higher PEG concentrations there was a decrease in quercitrin levels. Similar to the research of [31] that the optimum concentration of 3% PEG increases terpenoid levels in *Spilanthes acmella* Murr callus, then decreases with increasing PEG concentration. This can be due to the high concentration of PEG resulting in barriers to growth rates (tissue death) and plant metabolism. Gueven [32] states that elicitation will be effective in line with the exponential growth phase where plant metabolism reaches its maximum level.

The secondary metabolites contained in plants can be influenced by the concentration of elicitor given. Namdeo [33] suggested that elicitor concentrations can affect cell sensitivity responses in plant cultures. The response starts from the introduction of the elicitor (effector) by its receptors until it continues the signal to induce the production of secondary metabolites. After binding effectors with receptors occurs, the performance of the elicitor will decrease along with the growth period of the culture so that it has an impact on decreasing the secondary metabolite content, the event is called the post binding effect. Decreased quercitrin levels can be caused by a lack of receptors on plant cell surfaces when binding to elicitors above 10 ppm PEG. Estrada *et al.* [34] states that there are three mechanisms of cell sensitivity to elicitor, namely the number of elicitors given to receptors, the number of receptors on cells, and the process after binding between receptors and effectors. According to [13] and [35], the elicitor can work optimally on the treatment medium influenced by several factors such as elicitor type, elicitor concentrations, duration of elicitor contact, time course of elicitation, cell line, nutrient composition, and age or growth stage of the culture.

In general, PEG has an effect on increasing the compound content of quercitrin levels in *Chrysanthemum* buds compared to the control treatment. If a plant is under stress conditions, it will spontaneously (naturally) defend itself by producing secondary metabolites. In accordance with the statement of [17], secondary metabolites are a specific form of adaptation to changes in environmental conditions and act as a form of defense mechanism. Plants will drain energy allocated to growth into secondary metabolite production as a form of defense. Research by [15] and [34] said that the increase in the content of quercetin in cultures treated elicitor was due to elicitor interactions with plant receptors in activating second messengers, activation of gene responses, production of phytoalexin compounds, and accumulation of secondary metabolites.

Table 2. Average quercitrin content of *C. morifolium* Ramat shoots at various PEG concentration

PEG Concentration	Quercitrin content (mg/g DW)	Increased quercitrin levels
Control (0 ppm)	0.0062 b	-
10 ppm PEG	0.0275 b	343.55%
20 ppm PEG	0.0122 b	96.77%
30 ppm PEG	0.0115 b	85.48%
40 ppm PEG	0.0083 b	33.87%

Note: The mean value followed by the same letters showed no significant difference ($P < 0.05$).

Relation of growth and quercitrin content

The relation between growth patterns of *Chrysanthemum* shoots (*C. morifolium* Ramat) and quercitrin levels in various PEG concentrations was tested by Pearson Correlation Analysis (Figure 4). The growth variable tested was plantlet dry weight, because the parameter is a representative form of overall growth parameters. Results of Pearson Correlation Analysis showing sig. F change value of 0.013. This indicates that shoot growth is positively correlated with quercitrin levels. If sig. F change value <0.05 so it shows a correlation. The closeness of the correlation between shoot growth and quercitrin levels can be seen from the R. coefficient value. The value of the correlation coefficient (R) between the two parameters is 0.905 which means that it is very strongly correlated.

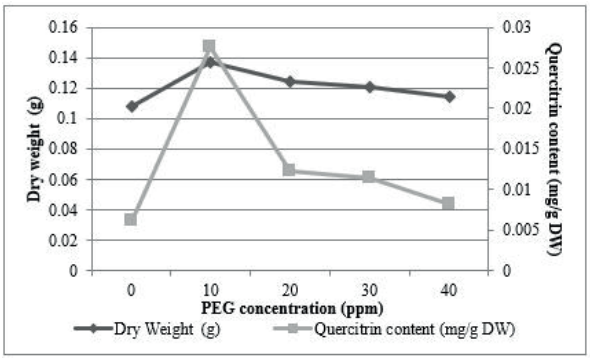

Figure 4. Correlation of plant growth with quercitrin content on various PEG concentration

The correlation between shoot growth and quercitrin content was then made in the form of a curve as shown in Figure 5.

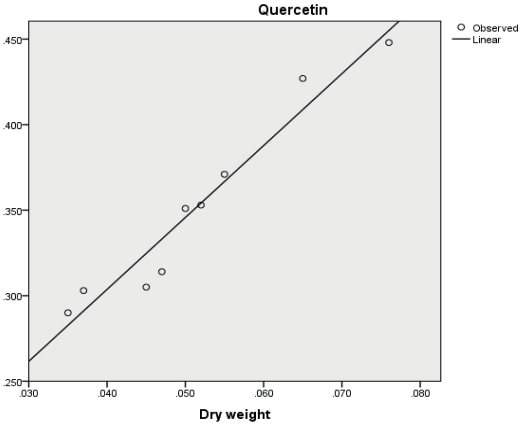

Figure 5. Linear regression curve of plant growth to quercitrin content

Figure 5 shows that shoot growth lines and quaternitrine levels have a similar pattern. This shows that quercitrin synthesis is in line with the growth of *Chrysanthemum* shoots. Quercitrin synthesis which takes place almost simultaneously with growth is thought to be related to the availability of precursors as a result of the primary metabolites needed in the biosynthesis of secondary metabolites. According to [29] and [9], giving certain concentrations of PEG to media in vitro can increase the dissolved sugar content in plants, if the PEG concentration increases, the dissolved sugar content will decrease. The dissolved sugar is one form of primary metabolite which will be used as a biosynthesis of secondary metabolites.

The biosynthetic pathway of secondary metabolites is divided into several groups according to the product produced. The sugar contained in *Chrysanthemum* shoot extracts is converted into cyclic acid, as a quercitrin precursor in the flavonoid biosynthetic pathway. Stracke *et al*. [36] suggested that quercitrin is one of the main flavonol glycosides from flavonoids.

Quercitrin levels decreased with increasing PEG concentration above 10 ppm. Decreased quercitrin levels can be caused by a decrease in the exponential phase of growth of *Chrysanthemum* shoots. Gueven [32] states that certain concentration elicitors can work optimally at the exponential phase of the plant, and then continue to decline in line with the plant growth curve. Decreasing quercitrin levels can be caused by competition in obtaining precursors. Murthy *et al*. [9] stated that in the metabolic process it is possible for competition to convert precursors to the growth process or to become a material for the production of secondary metabolites by utilizing enzymes.

Conclusion

Addition of PEG has a positive effect on growth of shoot height and quercitrin levels on shoot culture *of C. morifolium* Ramat cv. Yulimar. Shoot culture produced the highest quercitrin level of 0.02749 mg / g BK at 10 ppm PEG. The growth of shoots (dry weight of microshoots) correlated very strongly (R = 0.905) with quercitrin levels in shoot culture of *C. morifolium* Ramat cv. Yulimar.

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