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The effect of light and growth regulators on callus induction and growth in *Erythrina lithosperma Miq*

Paryatin Paryatin [1], Edy Setiti Wida Utami [1,*], Y. Sri Wulan Manuhara [1] and Pratiwi Pujiastuti [2]

[1] *Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indonesia.*
[2] *Department of Chemistry, Faculty of Science and Technology, Universitas Airlangga, Surabaya 60115, Indonesia.*

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Abstract

Erythrina lithosperma Miq is a plant that is traditionally used in medicine, with its leaves containing alkaloids such as erythramine, erysodine, erysopine, and hypaphorine. These compounds are known for properties that make them act as antipyretic, anti-inflammatory, and antimalarial agents. However, getting the raw materials for malaria drugs from natural sources can lead to issues like a loss of germplasm of the medicinal plants. In order to address such a challenge, biotechnology, particularly *in vitro* callus culture is necessary in providing a viable method for producing raw materials for malaria medicine. It is known that growth regulators can make callus form, and the *in vitro* method allows the media to be manipulated to get bioactive compounds with better quality, more quantity, better control, and greater stability. Therefore, this research aimed to investigate induction and proliferation of callus under various concentrations of growth regulators, specifically 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-Benzylamino-purine (BAP) in both dark and light conditions. The results showed that culturing in dark conditions with a combination of 1.0 mg/L 2,4-D and 0.5 mg/L BAP was the best for induction and proliferation of *E. lithosperma Miq.* This treatment produced the fastest and most substantial callus growth, with callus induction occurring 9 days after culture. The wet weight of callus was 1.268 g, and the dry weight was 0.082 g, which was higher than those observed with other treatments.

Keywords: *E. lithosperma Miq;* Callus growth and induction; Dark; Light; 2,4-D; BAP

1. Introduction

Malaria is still a serious health issue in both sub-tropical and tropical countries. The World Health Organization (WHO) estimates that 2.3 billion people or 41% of the world's population, live in areas at risk of malaria infection. Each year, approximately 300 – 500 million cases of malaria occur, resulting in an estimated 1.5 to 2.7 million deaths. In 2008, data showed that 109 countries were declared endemic areas for malaria. Indonesia is ranked 26th among countries in the world where malaria is endemic, with a prevalence of around 9.198 cases per 100.000 people [1].

Since 1863, malaria infection has been treated with quinine extracted from the bark of the Cinchona tree. However, the morbidity and mortality rates of the infection remain high in some regions due to factors such as the emergence of drug-resistant malaria parasite strains and insecticide-resistant Anopheles mosquitoes.

Currently, there is a shift in public interest toward natural ingredients, as they are believed to have a milder side effect and are relatively easy to obtain in the market. Indonesia has around 30.000 species of plants with therapeutic (curing) properties, but only 180 have been used traditionally. One such medicinal plant is coral tree (*Erythrina lithosperma Miq*) [2], which has properties similar to those of *Artemisia annua*, a known anti-plasmodium plant. In traditional medicine, various parts of *E. lithosperma Miq,* including the bark and leaves, are used. The leaves contain alkaloids such as

* Corresponding author: Edy Setiti Wida Utami

erythramine, erysodine, erysopine, and hypaphorine [3, 4], which have antipyretic, antimalarial, and anti-inflammatory properties, making the plant a promising candidate for malaria treatment.

A significant obstacle to using *E. lithosperma Miq* as a raw material for malaria treatment is its limited availability, along with the cultivation methods and the process of producing the active ingredient not being disclosed. Relying on natural sources for these raw materials can cause severe problems, such as the loss of germplasm from medicinal plants. Currently, a considerable portion of herbal ingredients distributed in Indonesia are being imported, showcasing the need for biotechnology interventions to address this problem.

Biotechnology, particularly *in vitro* callus culture through tissue culture methods, offers a promising solution for generating raw materials for malaria medicine. The method allows for the mass production of active compounds using bioreactor systems, ensuring consistent quality standards that can be both maximized and controlled. Within *in vitro* culture, callus can be developed into a cell suspension culture and allows the engineering of the media to obtain bioactive compounds with higher quality [5, 6, 7]. In terms of potential applications, this research becomes relevant to the government initiative to mitigate the limited supply of medicinal raw materials derived from natural ingredients. The success of callus culture depends on several cultures such as the basic media composition [8], the combination and concentration of added growth regulators [9, 10], appropriate environmental conditions, and the type of explant used [11]. The primary factors influencing *in vitro* plant growth are the interaction and balance between endogenous and exogenous plant growth regulators in cultured cells [12].

2. Material and methods

2.1. Source of explants and sterilization process.

This research was conducted at the Plant Physiology Laboratory, Department of Biology, Faculty of Science and Technology, Airlangga University, Surabaya, Indonesia. *Erythrina lithosperma Miq* was used as the plant material, specifically the 2nd and 3rd-order leaves of the shoot. The process of sterilizing explants was carried out in the following stages. The leaves, at first, were washed with running water, mainly to remove any dust that might be on them. After that, they were soaked in a detergent solution for 3 minutes and rinsed three times with sterile distilled water. The leaves were further soaked in a fungicide solution with 0.5 g/L mancozeb 80% (Dithane M-45 80WP) and rinsed again three times with sterile distilled water. Finally, they were sterilized in a 5% sodium hypochlorite (Clorox) solution for 5 minutes while shaking. The sodium hypochlorite solution was then discarded and rinsed three more times with sterile distilled water.

2.2. Callus induction and growth

After the process of sterilization, the leaves were put in a petri dish on top of filter paper and then cut with a scalpel into pieces about 1 cm^2. To evaluate the effect of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and Benzyl Amino Purine (BAP) on the induction and growth of *E. lithosperma Miq*, explants were grown in Murashige and Skoog media supplemented with four combinations of growth regulators, including 1.0 mg/L 2,4- D + 1.0 mg/L BAP, 1.0 mg/L 2,4-D + 0.5 mg/L BAP, 0.5 mg/L 2,4-D + 1.0. mg/L BAP, and 0.5 mg/L 2,4-D+ 0.5 mg/L BAP. The culture bottles were then stored in an incubation room at a temperature of 23 ± 2^0C, with samples maintained under both light (light intensity of ± 1.110 lux) and dark conditions (no light). To monitor callus formation, the induction period (in days) was recorded, beginning from the day after explants were planted. Observations of callus wet and dry weights were carried out at 3-week intervals, specifically at the 3rd, 6th, 9th, and 12th weeks after planting. The wet weight was determined by weighing the fresh callus tissue, excluding the bottle and planting medium, with measurements repeated 3 times for accuracy. Meanwhile, the dry weight was determined by placing callus in an incubator oven set to 60-70^{o}C.

2.3. Statistical analysis

The experiment followed a completely randomized design (CRD). Data were analyzed through the use of SPSS software (Version 24), using ANOVA to determine significance. The mean values were compared using the Mann-Whitney test at a significance level of ≤ 0.05.

3. Results

The average time required for callus induction under various treatments of growth regulators in both light and dark conditions was presented in Table 1. Under light conditions, callus formation occurred during the 3rd week, with the earliest induction time recorded on the 16th day for the 1.0 mg/L 2,4-D + 0.5 mg/L BAP treatment. Meanwhile, under

dark conditions, callus formed more quickly, with the earliest induction occurring in the 2nd week, specifically on the 9th day for the 1.0 mg/L 2,4-D + 0.5 mg/L BAP treatment.

Table 1 Average callus induction time in various growth regulators treatments in light and dark conditions.

Plant growth regulators concentrations (mg/L)		**The mean time explants to form callus (day)**	
2.4-D	BAP	light	dark
1.0	1.0	20.00 ± 1.31^{e}	11.00 ± 1.06^{b}
1.0	0.5	16.00 ± 1.29^{d}	9.00 ± 0.00^{a}
0.5	1.0	20.00 ± 1.22^{e}	11.00 ± 0.90^{b}
0.5	0.5	21.00 ± 1.24^{f}	12.00 ± 0.79^{c}

Mean±SD followed by the same letter was not significantly different at the P 5% Mann Whitney test.

The average wet weight of callus after culturing at the 3rd, 6th, 9th, and 12th weeks with growth regulators (2,4-D and BAP) in light and dark conditions was presented in Table 2.

Table 2 Average wet weight of callus given a combination of growth regulators (2,4-D and BAP) in light and dark conditions

Growth regulator concentrations (mg/L)		**Wet weight of callus (g)**							
2.4-D	BAP	3rd week		6rd week		9rd week		12rd week	
		light	dark	light	dark	light	dark	light	dark
1.0	1.0	0.067 ± 0.007^{a}	0.073 ± 0.009^{a}	0.096 ± 0.015^{a}	0.338 ± 0.166^{b}	0.080 ± 0.006^{a}	0.824 ± 0.132^{d}	0.431 ± 0.051^{c}	1.211 ± 0.194^{gh}
1.0	0.5	0.057 ± 0.004^{a}	0.134 ± 0.027^{a}	0.103 ± 0.015^{a}	1.162 ± 0.072^{g}	0.129 ± 0.023^{a}	1.185 ± 0.167^{g}	0.130 ± 0.034^{a}	1.268 ± 0.121^{h}
0.5	1.0	0.069 ± 0.007^{a}	0.077 ± 0.007^{a}	0.081 ± 0.008^{a}	0.473 ± 0.065^{c}	0.080 ± 0.009^{a}	1.022 ± 0.050^{f}	0.276 ± 0.071^{b}	1.134 ± 0.147^{g}
0.5	0.5	0.071 ± 0.005^{a}	0.071 ± 0.004^{a}	0.072 ± 0.003^{a}	0.810 ± 0.239^{d}	0,082 ± 0.007^{a}	0.887 ± 0.052^{e}	0.270 ± 0.079^{b}	0.998 ± 0.131^{f}

Mean ± SD followed by the same letter was not significantly different at the P 5% Mann-Whitney test.

Table 2 showed that the highest average wet weight of 1.268 ± 0.121 g was obtained in the treatment of 1.0 mg/L 2.4-D + 0.5 mg/L BAP (dark) at 12 weeks after culture.

The average dry weight of callus after culturing for the 3rd, 6th, 9th, and 12th weeks with growth regulators (2,4-D and BAP) in light and dark conditions was presented in Table 3.

Table 3 Average dry weight of callus given a combination of growth regulators (2,4-D and BAP) in light and dark conditions.

Growth regulator concentrations (mg/L)		**Dry weight of callus (g)**							
2.4-D	BAP	3rd week		6rd week		9rd week		12rd week	
		light	dark	light	dark	light	dark	light	dark
1.0	1.0	0.007± 0.001^{ab}	0.007± 0.001^{ab}	0.010± 0.003^{abcd}	0.031± 0.011^{f}	0.008± 0.001^{abc}	0.058± 0.011^{h}	0.032± 0.005^{fg}	0.069± 0.011^{i}

1.0	0.5	0.006± 0.000^{a}	0.013± 0.002^{cd}	0.010± 0.001^{abcd}	0.084± 0.009^{j}	0.015± 0.002^{d}	0.085± 0.011^{j}	0.012± 0.003^{bcd}	0.082± 0.006^{j}
0.5	1.0	0.007± 0.001^{ab}	0.007± 0.001^{ab}	0.008± 0.001^{abc}	0.036± 0.006^{g}	0.008± 0.001^{abc}	0.066± 0.004^{i}	0.023± 0.008^{e}	0.066± 0.009^{di}
0.5	0.5	0.007± 0.001^{ab}	0.007± 0.001^{ab}	0.007± 0.000^{abc}	0.057± 0.007^{h}	0.008± 0.001^{abc}	0.064± 0.005^{i}	0.023± 0.006^{e}	0.058± 0.010^{h}

Mean ± SD followed by the same letter is not significantly different at the P 5% Mann-Whitney test.

The highest average dry weight of callus was obtained in the treatment of 1.0 mg/L 2.4-D + 0.5 mg/L BAP after 9 weeks of culture in dark conditions, namely 0.085 ± 0.01 g.

Callus morphology of *E. lithosperma* leaf explants with a combination of growth regulators (2,4-D and BAP) under light conditions is presented in Figure 1.

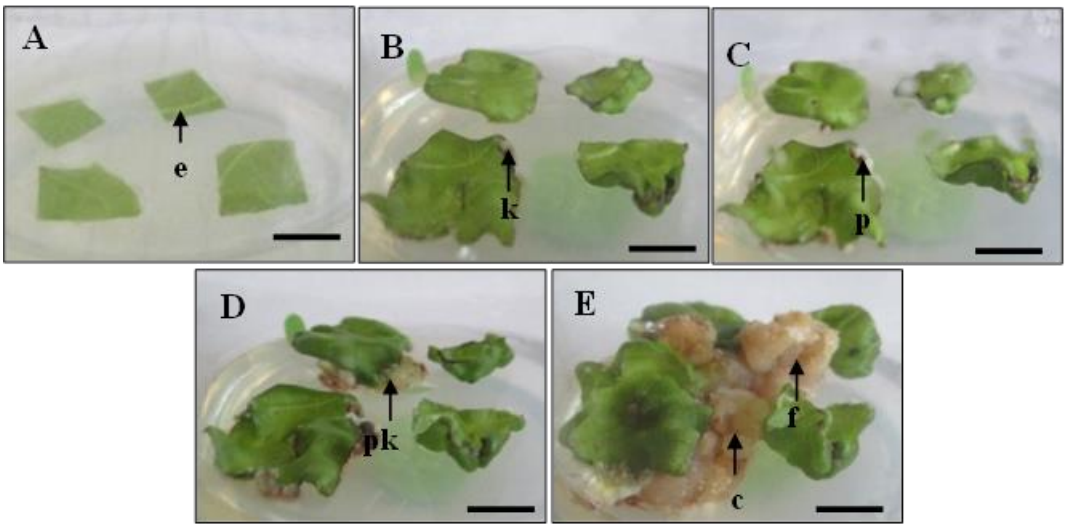

Figure 1 Callus morphology in the treatment of 1.0 mg/L 2,4-D + 0.5 mg/L BAP under light conditions. A = explants when cultured, B = 3 weeks after culture, C = 6 weeks after culture, D = 9 weeks after culture, E = 12 weeks after culture. e = explant, k = callus, p = white, pk = brownish white, c = brown, f = friable (Bar = 1.15 cm).

In the 3rd week, the explants became swollen and increased in size, had dark green color, and callus began to appear on the wound, as presented in Figure 1.

The morphology of callus from *E. lithosperma* leaf explants with a combination of growth regulators (2,4-D and BAP) in dark conditions were presented in Figure 2.

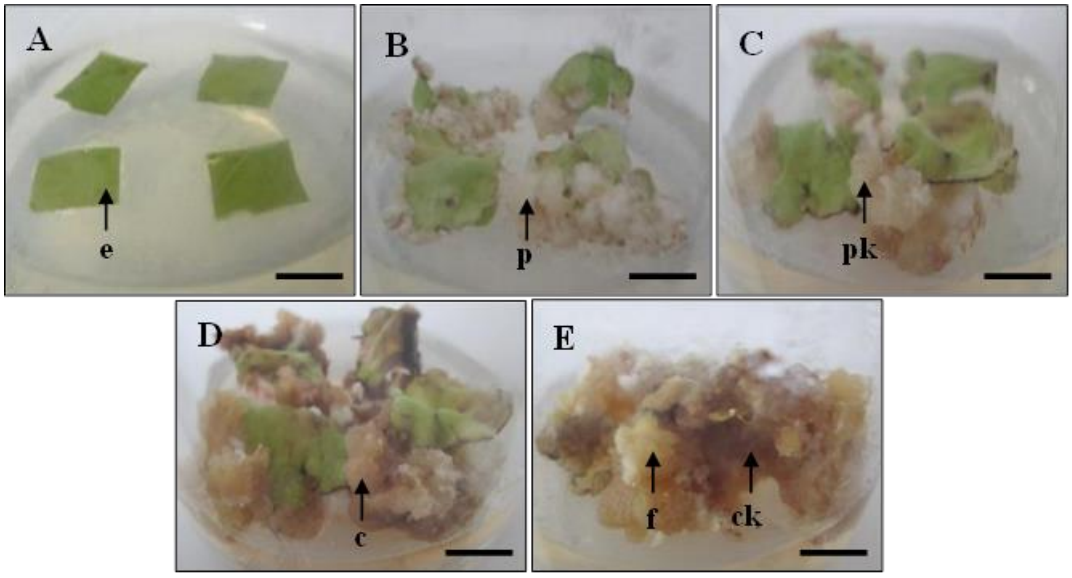

Figure 2 Morphology of callus treated with 1.0 mg/L 2,4-D + 0.5 mg/L BAP in dark conditions. A = explants when cultured, B = 3 weeks after culture, C = 6 weeks after culture, D = 9 weeks after culture, E = 12 weeks after culture. e = explant, k = callus, p = white, pk = brownish white, c = brown, ck = blackish brown, f = friable, (Bar = 1.15 cm)

Figure 2 showed that in the 3rd week, the explants swelled and increased in size, had light green color, and developed callus quickly.

4. Discussion

4.1. Effect of 2.4-D and BAP on callus induction and morphology.

The observation results of effect of 2,4-D and BAP on callus induction showed that all growth regulators treatments (1.0 mg/L 2.4-D + 1.0 mg/L BAP, 1.0 mg/L 2.4-D + 0.5 mg/L BAP, 0.5 mg/L 2.4-D + 1.0 mg/L BAP, and 0.5 mg/L 2.4-D + 0.5 mg/L BAP) successfully induced callus formation under both light and dark conditions. Callus formation occurred more rapidly in dark conditions, initiating in the 2nd week after planting, compared to the 3rd week in light conditions.

The Mann-Whitney significant test on the average time required for callus formation under light conditions showed that the 1.0 mg/L 2,4-D + 0.5 mg/L BAP was significantly different from the other treatments. This treatment had the fastest callus induction, with explants forming callus by the 16th day. Based on the results of the Mann-Whitney significant difference test, in dark conditions, the concentration of 1.0 mg/L 2,4-D + 0.5 mg/L BAP also showed a significant difference from the other treatments. The treatment had the fastest callus induction, with explants forming callus by the 9th day. Callus induction happened both with the presence or absence of light. In many plants like chili peppers [13], tomatoes [14], and tobacco [15], callus forms better with light. But in this case, the analysis showed that dark conditions seemed more suitable for inducing callus in *E. lithosperma* leaf explants. This aligned with an observation made by Yanjie C [16], who reported that for *Nicotiana tabacum*, a higher percentage of leaf explants formed callus in dark conditions (100%) compared to those in light conditions (80%). Similarly, Karunaratne [17] obtained the highest callus induction results from teak (*Tectona grandis*) leaves at 96% under dark conditions. Khan *et al* (18) observed a similar phenomenon in *Citrus reticulata* callus induction using hypocotyl and epicotyl explants.

The results of the observations in light conditions showed that in the 3rd week after culture, the explants swelled, with an increase in size and a green coloration, and callus formation began at the wound site (Figure 1B). This response was likely due to the influence of auxin in the media and turgor pressure. The presence of auxin promotes cell wall stretching and loosening, which occurred as acid secretion activated certain enzymes. The enzymes would break the bonds between cellulose molecules in the cell walls, leading to cell elongation. Turgor pressure occurred when cells absorb water molecules in response to the increasing concentration of solutes contained in the vacuole, facilitating further cell expansion [19]. In the 6th week after culture, callus began to develop and appeared white (Figure 1 C). At the 9th week, callus color changed to brownish-white (Figure 1 D), and in the 12th week, it turned brown (Figure 1 E). In all treatments, callus had a friable texture, indicating that it broke apart easily (Figure 1 E).

In dark conditions, observations showed that by the 3rd week of culture, the explants were swollen, appeared light green, and had highly developed and white callus (Figure 4.2 B). The light green color was influenced by the auxin activity in promoting callus growth. In the 6th week, callus had turned brownish-white, as detailed in Figure 2 C. At the 9th week of culture, it became brown (Figure 2 D) and then dark brown (Figure 2 E) at the 12th week of culture. In these cultures, callus regions that continued dividing remained white, even though some areas were light brown. All treatments under dark conditions also showed a friable callus texture, as detailed in Figure 2 E. During this research, there was visible browning of callus originating from the leaves in both dark and light conditions (Figures 1 and 2). The browning showed the presence of polyphenolic compounds in the leaves sections used for callus initiation.

4.2. Effect of 2.4-D and BAP on fresh and dry weight callus

The wet and dry weights of callus served as key indicators of its growth. The observation results of effect of 2,4-D and BAP showed that all growth regulators treatments (1.0 mg/L 2,4-D + 1.0 mg/L BAP, 1.0 mg/L 2,4-D + 0.5 mg/L BAP, 0.5 mg/L 2,4-D + 1.0 mg/L BAP, and 0.5 mg/L 2,4-D + 0.5 mg/L BAP) could increase both wet and dry weights of callus (Tables 2 and 3) under light and dark conditions. According to Chawla [20], George et al [21], and Park *et al* [22], auxin was widely used for callus induction due to its role in cell division, cell elongation, vascular tissue differentiation, as well as rhizogenesis, root formation, embryogenesis, and inhibition of axillary shoot growth. Cytokinin, a derivative of Adenine, was essential for regulating the synthesis of proteins that facilitated spindle fiber formation during mitosis [20, 21]. It was also required for the formation of adventitious buds and the stimulation of cell division [20, 21]. In general, the types of cytokinin used in plant tissue culture practice included BA, BAP, zeatin, kinetin, and most recently TDZ.

The results from the analysis showed how growth regulators affected callus growth, mainly depending on the concentration of the regulator. The 1.0 mg/L 2,4-D combined with 0.5 mg/L BAP emerged as the most effective concentration for callus induction and growth. In particular, media supplemented with this concentration under dark conditions achieved the highest results (wet weight: 1,268 grams and dry weight: 0.08 grams) at week 12 after culture. This showed that callus, when treated with 1.0 mg/L 2,4-D + 0.5 mg/L BAP, could grow in dark conditions, where cells apparently optimized their metabolism during the growth period, making wet and dry weights higher. The results suggested that 1.0 mg/L 2,4-D + 0.5 mg/L BAP concentration under dark conditions was somehow the best for more proliferated callus compared to other treatments.

5. Conclusion

In conclusion, adding 2,4-D and BAP growth regulators has a strong impact on how callus formed and grew. Specifically, using 1.0 mg/L 2,4-D + 0.5 mg/L BAP in dark conditions turned out to be most effective for making callus from *E. lithosperma* Miq leaf explants grow. Morphologically, callus grown under various 2,4-D and BAP concentrations in light conditions showed colors from white to brownish-white and brown. Under dark conditions, callus colors ranged from white to brownish-white to brown and even blackish-brown. Across all treatments, callus consistently had a friable texture, which means it could disintegrate easily.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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