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Research

The Effects Of 2,4-D, BAP, and Sucrose Concentrations in The Callus Induction of White (*Clitoria ternatea* var. *Albiflora*) and Blue Butterfly Pea (*Clitoria ternatea*)

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ABSTRACT

The blue butterfly pea (*Clitoria ternatea*) and white butterfly pea (*Clitoria ternatea* var. Albiflora) belong to the Fabaceae family. Both are locally known as “bunga telang” and native to the Southeast Asian regions. The blue flowered variety is traditionally used to treat headaches, fever, and diabetes and is renowned scientifically for its memory-enhancing properties due to the presence of novel pentacyclic triterpenoids. However, farming of *C. ternatea* is challenged by inconsistent yields of novel secondary metabolites, especially under changing environmental conditions. Callus and cell suspension cultures, on the other hand, offer an alternative for the consistent production of these metabolites. The current study aims to optimize the treatments of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), and sucrose concentrations for friable callus formation from seedling explants. Sterile cotyledon explants of *in vitro* seedlings from both types of butterfly pea were subjected to half-strength MS medium supplemented with different concentrations and combinations of 2,4-D and BAP, with sucrose at 15 g/L and 30 g/L. The highest friable callus fresh weight from the white butterfly pea explants (0.064 ± 0.010 g) was achieved in treatments of 0.40 mg/L 2,4-D and 0.50 mg/L BAP. In contrast, the highest fresh weight of friable callus for the blue variety (0.025 ± 0.016 g) was induced in 0.25 mg/L of 2,4-D. Both varieties showed the highest friable callus weight in 15 g/L sucrose supplemented with 1.00 mg/L of 2,4-D (0.146 ± 0.032 g) and 0.25 mg/L of 2,4-D (0.245 ± 0.075 g) for the white and blue variety respectively. The morphology of calli for both varieties were yellowish, watery, and sticky. This study provides an essential basis the establishment of cell suspension cultures, as an efficient alternative to harness the secondary metabolites associated with the mammalian neuroprotective properties.

Key words: Butterfly pea, friable callus, sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP)

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INTRODUCTION

Clitoria ternatea L. is a legume plant from the Fabaceae family and is commonly known as "bunga telang" in Malaysia, and Aparajita in Bengali. It is native to tropical Asia, including Malaysia, India, the Philippines, and Thailand. This perennial climber can grow up to 6 meters tall with a bushy structure, valued for its decorative aspects (Jamil & Pa’ee, 2018). The plant produces individual, axillary, papilionaceous flowers, with a calyx comprising five fused sepals and a corolla featuring five separate petals. In Asia, its petals are traditionally used as a natural food coloring and tea. For instance, in Malaysia, the flower adds a vibrant blue color to traditional dishes like nasi kerabu, pulut inti, and pulut taitai. Additionally, after dinner, blue butterfly pea tea is often served with lemon and honey as a refreshment at hotels or spas in Thailand and Vietnam. The acidity of the lemon changes the tea's color from blue to purple (Goldberg, 2016).

Moreover, *C. ternatea* could serve as a cover crop due to its capacity to fix nitrogen and enhance soil quality (Gupta *et al*., 2010; Mohamed & Taha, 2011). According to Mukherjee *et al*. (2008), *C. ternatea* also has a large number of thin lateral roots that grow into powerful taproot branches.

The woody, fresh root of *C. ternatea* has an unpleasant taste and pale color with a slight bitterness. Traditionally, the plant's roots, leaves, petals, and seeds are utilized in Indian Ayurvedic medicine to treat various ailments, such as anthelmintic properties, an antidote for animal stings, a laxative and an inflamed joint (Mukherjee *et al*., 2008; Dighe *et al*., 2009; Al-Snafi, 2016). Additionally, the root of *C. ternatea* is employed in treating conditions such as asthma, leprosy, and inflammation (Mukherjee *et al*., 2008; Dighe *et al*., 2009). Interestingly, the root with honey or ghee was given to children to improve memory.

Clitoria ternatea can be categorized into different varieties, including the white variety, scientifically known as *C. ternatea* var. Albiflora, and the blue variety are commonly studied (Figure 1). Both varieties share similarities, with slight differences noted in the blue variety, as previous research has indicated that morphologically, both blue and white varieties are almost identical, except for the color of the petals (Khatoon *et al*., 2015). This report also indicated that the macroscopic analysis of *C. ternatea* revealed unique anatomical features such as increased starch grains in the transverse section of the root, vast patches of pericyclic fibers, a higher number of vessels with broad lumens, and a broad pith region in the stem of the white variety. Additionally, quantitative leaf microscopy indicated slight variations in stomatal number, stomatal index, and palisade ratio between both varieties, which were observed higher in the white variety (Khatoon *et al*., 2015). This report also indicated the white variety contains higher levels of flavonoids, alkaloids, carbohydrates, and glycosides than the blue variety. On the other hand, the blue variety has higher concentrations of tannins and saponins, and both varieties exhibit similar levels of triterpenoids and steroids (Khatoon *et al*., 2015). Furthermore, Kumar and Dhobi (2017) found that the blue variety exhibits more potent anti-anxiety and antioxidant properties than the white ones. In addition, the nutritional content differed between the two varieties. Turnos (2021) demonstrated that the blue variety has higher levels of nutrients such as Phosphorus (P), nitrogen (N), calcium (Ca), Magnesium (Mg), Potassium (K), ash content (inorganic), and dry matter. Even though the bio profiles for both varieties are slightly different, they exhibited the same pharmacological properties, which are antioxidant, anti-anxiolytic, anti-microbial, and anti-anxiety properties (Kumar & Dhobi, 2016; Kumar & Anju, 2017; Divya *et al*., 2018).

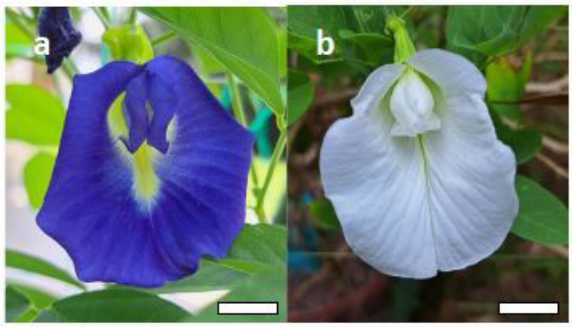

Fig. 1. The variety of *Clitoria ternatea* flowers. **(a)** Blue butterfly pea (*C. ternatea*), **(b)** White butterfly pea (*C. ternatea* var. albiflora). The scale bar represents 1 cm.

Nowadays, *C. ternatea* has become the center of attention among researchers due to its neuroprotective properties' potential as new drugs for neurodegenerative diseases such as dementia or Alzheimer's disease. As mentioned, Damodaran *et al*. (2020) demonstrated that methanolic extracts of *C. ternatea* roots may improve cognitive impairment to promote the function of memory and learning while restoring memory in a rat study. With reference to Rai (2010), the aqueous extract of root *C. ternatea* as a growth promoter of neurons have been studied, where they reported that the extracts stimulated the growth of neural stem cells at the anterior subventricular zone, (aSVZ). Turnos (2021) on the other hand also discovered both varieties contain taraxerol and taraxerone, the pentacylic triterpenoids mainly found in roots linked to memory-enhancing properties. However, the butterfly pea's smaller root mass may lead to challenges and inconsistent production of secondary metabolites using conventional cultivation methods (Lee *et al*., 2021). According to Mukherjee *et al*. (2008), only 12.4 mg of taraxerol per gram of root extract has been extracted, whereas Lee *et al*. (2021) demonstrated that 6.35% of taraxasterol can be obtained from 2 mg of ethanolic root extracts. This has caused much strain on the metabolite production from conventional farms when plant secondary metabolites become highly expensive to meet the demands (Al-Asmari *et al*., 2020). Plant tissue culture technology is a reliable option that has been long established for consistently producing novel plant secondary metabolites under controlled environments. Callus cultures function as the starting material for the propagation

of plant cells and the production of phytochemical compounds without the concern of overharvesting *C. ternatea* (Rai *et al*., 2022). These properties allow for the quick generation of clones that closely resemble their mother cells and preserve their genetic makeup (Verdeil *et al*., 2007). Callus and cell suspension cultures have been widely used up to this point to produce novel phytochemical substances from medicinal plants such as *Eurycoma longifolia* (Tongkat Ali), *Taraxacum officinale* Weber, and *Artemisia annua* L (Chan *et al*., 2010; Sharma & Zafar, 2016; Nhan & Loc, 2017). The production of taraxerol and taraxasterol from *Taraxacum officinale* cultures was previously reported to be successful (Sharma & Zafar, 2016), further indicating the ability of cell cultures to produce such metabolites. The biosynthesis of taraxerol starts from the precursor squalene that is commonly found in plant cell wall and parenchyma cells that leads to the formation of (3S)-2,3-epoxy-2, 3-dihydrosqualene, dammarenyl cation, baccharenyl cation, lupanyl cation, and olean-19-yl-cation, olean-19-ylcation further forms taraxerol and taraxasterol (Sharma & Zafar, 2015).

Thus, the callus and cell suspension cultures are reliable alternatives in the induction and accumulation of novel secondary metabolites for large-scale production. Up to this point, literary evidence on the cell suspension cultures for both varieties of *C. ternatea,* especially the white-flowered variety, is scarce. Previous studies highlighted the *in vitro* regeneration of blue butterfly pea plant, such as adventitious root cultures, callus induction, and root induction (Mohamed & Taha, 2011; Chan *et al*., 2017; Mishra *et al*., 2019; Lee *et al*., 2021; Doffek *et al*., 2022; Teoh *et al*., 2023). Hence, this current study aims to evaluate callus regeneration between both varieties of butterfly pea via treatments in different sucrose concentrations, and in the combination treatments of 2,4-D and BAP. This is an essential step for subsequent studies on the development of cell suspension cultures and the synthesis of new secondary metabolites from *C. ternatea*.

MATERIALS AND METHODS

Plant material

The mature seedpods of blue butterfly peas were harvested from the outdoor mother plant cultivated at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia. The seeds were rinsed for 15 min under running tap water. Subsequently, the seeds were surface sterilized in 60% v/v Clorox© (bleach) solutions with two drops of Tween 20 for ten min under mild agitation, followed by one min of 70% v/v ethanol treatment in aseptic condition. After that, the seeds were washed three times with autoclaved distilled water and dried on sterilized filter paper. The surface-sterilized seeds were then placed on the half-strength Murashige and Skoog (1/2 MS) (Murashige & Skoog, 1962) free medium (without plant hormones) with 1.5% (w/v) sucrose and 0.8% plant agar powder (Duchefa Biochemie B.V., The Netherlands) followed by the incubation of cultures under the white fluorescent light (Philips TLD, 36W. 150µmol $m^{-2}s^{-2}$) about 16 hr photoperiod at the temperature of 25 ± 2°C. After two weeks, the sterile cotyledon explants were excised into the size of 0.6 × 0.6 cm^2 and subjected to hormone treatments subsequently. The steps were repeated for a white variety of plants.

Callus induction

The cotyledon explants were inoculated onto the half-strength MS medium supplemented with the combination of 2,4-D and BAP, as shown in (Table 1). The half-strength MS medium consisted of 3% (w/v) of sucrose and 0.8% of plant agar powder (Duchefa Biochemie B.V., The Netherlands). The experiments were repeated thrice, whereby each treatment contained nine samples with three explants per replicate. The experiments were conducted in a completely randomized design (CRD). The maintenance of cultures was placed under white, fluorescent light (Philips TLD, 36W. 150µmol m^{-2} s^{-2}) in a 16-hr photoperiod at the temperature of 25 ± 2°C. In the meantime, the steps were repeated for the sucrose concentration of 1.5% (w/v) and 3% (w/v). The medium used in this experiment was half-strength MS medium supplemented with different concentrations of 2,4-D only (0.25 mg/L, 0.5 mg/L, 0.75 mg/L and 1.0 mg/L) for each sucrose concentration.

Data collection and statistical analysis

Parameters such as the percentage of callus induction, fresh weight of callus, and morphology of callus were recorded after 60 days of incubation. The data collected were analyzed using one-way analysis of variance (ANOVA) at a 95% significance level via statistical analysis (IBM Statistical Package for the Social Sciences, SPSS) software version 27. Additionally, data for the parameter of callus fresh weight was subjected to comparison via T-test analysis with the control treatment.

Table 1. The combination of 2,4-D and BAP treatments for white and blue butterfly pea

White butterfly pea		Blue butterfly pea	
Treatment (mg/L)		Treatment (mg/L)	
2,4-D	BAP	2,4-D	BAP
0.00	0.00	0.00	0.00
0.20	0.00	0.25	0.00
0.20	0.10	0.25	0.25
0.20	0.50	0.25	0.50
0.40	0.00	0.25	0.75
0.40	0.10	0.25	1.00
0.40	0.50	0.50	0.00
0.60	0.00	0.50	0.25
0.60	0.10	0.50	0.50
0.60	0.50	0.50	0.75
0.80	0.00	0.50	1.00
0.80	0.10	-	-
0.80	0.50	-	-
1.00	0.00	-	-
1.00	0.10	-	-
1.00	0.50	-	-

RESULTS AND DISCUSSION

The effects of 2,4-D and BAP on friable callus induction for White and Blue Butterfly Pea plants.

The current study revealed that the white and blue butterfly pea plants had different responses toward the combination of plant growth regulators, which are 2,4-D and BAP. Friable callus was successfully induced in the combination of 2,4-D and BAP for the white variety, whereas its induction was not successful in the blue variety. Next, the results indicated that the optimal treatment in friable callus induction using cotyledon explants of white variety was 0.40 mg/L of 2,4-D combined with 0.50 mg/L of BAP as a 100% callus induction rate was achieved. In addition, this concentration managed to yield the highest fresh weight of 0.064 ± 0.010 g in the white variety, with significant differences between other treatments (0.8 mg/L 2,4-D and 0.1 mg/L BAP, 1 mg/L 2,4-D and 0.1 mg/L BAP and control) (Table 2). Meanwhile, for the blue variety, the optimal treatment for friable callus induction was adequate with treatments of 0.25 mg/L of 2,4-D alone, with a fresh weight of 0.025 ± 0.016 g. As for the blue variety, no significant differences were observed between the treatment of 0.25 mg/L of 2,4-D with all the treatments, but there was a significant difference between 0.5 mg/L of 2,4-D and 1.0 mg/L of BAP with the control (Table 3). However, the treatment of 0.25 mg/L of 2,4-D alone resulted in a significant difference with the control through T-test comparison. The type of callus obtained from both varieties in optimum treatments was yellowish, friable, sticky, soft, and watery (Figures 2 & 3).

Table 2. The effects of 2,4-D and BAP on callus induction from cotyledon explants of white butterfly pea plants. *N*=144

Treatment (mg/L)		Callus Induction (%)	Fresh Weight (g)	Callus Morphology
2,4-D	BAP			
0.00	0.00	25.0	0.001 ± 0.001^{e}	Friable
0.20	0.00	62.5	0.016 ± 0.006^{de}	Friable
0.20	0.10	100.0	0.049 ± 0.007^{abc}	Friable
0.20	0.50	75.0	0.036 ± 0.010^{abcd}	Friable
0.40	0.00	100.0	0.038 ± 0.004^{abcd}	Friable
0.40	0.10	100.0	0.038 ± 0.007^{abcd}	Friable
0.40	0.50	100.0	0.064 ± 0.010^{a}	Friable
0.60	0.00	87.5	0.032 ± 0.008^{bcd}	Friable
0.60	0.10	87.5	0.034 ± 0.010^{abcd}	Friable
0.60	0.50	75.0	0.061 ± 0.014^{ab}	Friable
0.80	0.00	100.0	0.039 ± 0.007^{abcd}	Friable
0.80	0.10	75.0	0.030 ± 0.017^{cd}	Friable
0.80	0.50	75.0	0.039 ± 0.011^{abcd}	Friable
1.00	0.00	75.0	0.040 ± 0.012^{abcd}	Friable
1.00	0.10	62.5	0.015 ± 0.005^{de}	Friable
1.00	0.50	100.0	0.036 ± 0.003^{abcd}	Friable

The data were presented as mean ± SE. Mean values followed by the same letter were not significantly different (Duncan test at $p \leq 0.05$)

Fig. 2. Callus induction from cotyledon explants of white butterfly pea plants in half-strength MS medium supplemented with 2,4-D and BAP after 60 days. **(a)** Control (0 mg/L), **(b)** 2,4-D (0.40 mg/L) + BAP (0.50 mg/L), and **(c)** 2,4-D (1.0 mg/L) + BAP (0.1 mg/L). The scale bar represents 1 cm.

Table 3. The effects of 2,4-D and BAP on callus induction from cotyledon explants of blue butterfly pea plants. *N*=99

Treatment (mg/L)		Callus Induction (%)	Fresh Weight (g)	Callus Morphology
2,4-D	BAP			
0.00	0.00	0.0	0.000 ± 0.000^{b}	N/A
0.25	0.00	100.0	0.025 ± 0.016^{ab*}	Friable
0.25	0.25	100.0	0.009 ± 0.004^{ab}	Compact
0.25	0.50	87.5	0.027 ± 0.008^{ab}	Compact
0.25	0.75	100.0	0.024 ± 0.001^{ab}	Compact
0.25	1.00	100.0	0.020 ± 0.007^{ab}	Compact
0.50	0.00	100.0	0.015 ± 0.010^{ab}	Friable
0.50	0.25	62.5	0.006 ± 0.002^{ab}	Compact
0.50	0.50	75.0	0.019 ± 0.008^{ab}	Compact
0.50	0.75	100.0	0.024 ± 0.008^{ab}	Compact
0.50	1.00	87.5	0.033 ± 0.011^{a}	Compact

The data were presented as mean ± SE. Mean values followed by the same letter were not significantly different (Duncan test at $p \leq 0.05$).* present mean ± SE Mean value was significantly different from control (T-test).

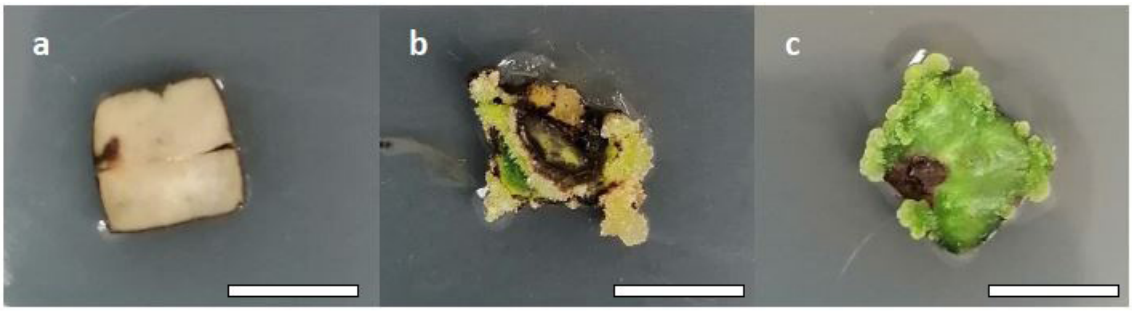

Fig. 3. Callus induction from cotyledon explants of blue butterfly pea plants in half-strength MS medium supplemented with 2,4-D and BAP after 60 days. **(a)** Control (0 mg/L), **(b)** 2,4-D (0.25 mg/L) + BAP (0 mg/L), and **(c)** 2,4-D (0.5 mg/L) + BAP (1.0 mg/L). The scale bar represents 1 cm.

Effective procedures for inducing friable callus in medicinal plants belonging to the Fabaceae family, like *Mucuna pruriens* (L.), *Sophora alopecuroides* (L.), and *Eysenhardtia platycarpa* (L.), and the synthesis of secondary metabolites enhanced by application of callus culture have been established (Senthil, 2020; Bernabé-Antonio *et al*., 2021; Rakesh & Praveen, 2022). On the other hand, a few studies reported the *in vitro* callus culture of *C. ternatea,* especially for the blue variety. For instance, Teoh *et al*. (2023) discovered that cotyledon explant was the suitable explant for friable callus induction in 2,4-D treatments. Other studies also reported that callus could be induced from cotyledon explants of *C. ternatea* via 2,4-D, indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA) treatments (Chan *et al*., 2017; Lee *et al*., 2021). These investigations, however, only examined on the blue variety. Therefore, this study evaluated both varieties to compare the differences in the plant secondary metabolite profiles between callus and cell suspension cultures.

In the present study, callus formation was identified from the excision sites of cotyledon at the fourth

week of incubation, and cotyledon explants started to expand. According to Park *et al*. (2023), plant chemicals called auxin and cytokinin are crucial in regulating the formation of callus. It is thought that the ratios of these hormones dictate the course of a cell's development and a higher auxin concentration to cytokinin typically promotes the production of roots, whereas a reversed ratio favors the regeneration of shoots. Thus, an appropriate proportion of auxin and cytokinin is typical in callus induction (Park *et al*., 2023). Auxin stimulates the formation of callus by activating auxin response genes such as lateral organ boundaries domain (LBD), which are mediated by the binding of auxin response factor (transcription factor) to the auxin-responsive element (AuxRE) (Ikeuchi *et al*., 2013; Song, 2014). The LBDs then activate the expression of genes to modify cell walls and promote cell proliferation. Hence, in the treatment of 2,4-D (a strong synthetic auxin), callus formation occurred when the tissues within the explants actively underwent cell division, resulting in the swelling of the explants. In addition, Majda and Robert (2018) discovered that the presence of auxin also causes the expansion and enlargement of explants via the synthesis of a proton pump leading to wall-loosening in an acidic environment. Next, the mechanism of callus formation using cytokinin is less clear, but the type B response regulator (B-RR) is a critical component participating in callus induction. The type B-RR promotes the cell cycle reentry to the potential target, cyclin-D (CYCD), and the overexpression of CYCD enhances the callus formation. Thus, the highly stable cytokinin BAP has a role in stimulating cell division and slowly degrades, which aids in callus formation (Normasari *et al*., 2023). Theoretically, this report indicated the combination of 2,4-D and BAP may act synergistically or antagonistically depending on the plant tissues' genotype and physiological condition to produce a high callus volume.

The outcome of this study indicated that the white and blue varieties have synergistic effects of 2,4-D and BAP in callus formation. However, the morphology of the callus obtained is different for both varieties in treatments with 2,4-D and BAP; in the white variety, the friable, soft callus was formed, whereas, in the blue variety, the compact, hard callus was formed, thus, not suitable for subsequent cell suspension cultures. Previous studies demonstrated the combination effects of 2,4-D, and BAP resulted in friable callus production from other species in the Fabaceae family such as *Parkia timoriana* (Thangjam & Maibam, 2006), *Tephrosia tinctoria* (Rajaram *et al*., 2013), and *Vigna aconitifolia* (Hande *et al*., 2015). In contrast, studies reported that combined effects of 2,4-D and BAP produced compact calli and 2,4-D alone is better in callus induction. These include species such as *Vigna subterranea* (Konate *et al*., 2013), and *Senna alata* (Lara *et al*., 2022). According to previous research on the blue butterfly pea plant by Lee *et al*. (2021) and Teoh *et al*. (2023), 2,4-D is sufficient to induce callus from cotyledon explants. These two studies align with the current study whereby the highest regeneration of callus formed at lower concentrations of single treatments of 2,4-D. Conversely, Chan *et al*. (2017) discovered that both NAA and IBA could regenerate callus, but the callus production by IBA was insignificant (0 to 20%), whereas high concentrations (1.5 mg/L) of NAA resulted in 100% callus formation. Hence, auxin alone is adequate to produce callus for the explants of the blue butterfly pea. With the exception of the control group, all of the treatments in this study had effectively produced callus for the blue variety, indicating that 2,4-D is necessary for friable callus induction, whereas the addition of BAP resulted in compact callus formation (Table 3). As for the control group, for the white variety, the cotyledon explants managed to induce callus (25%), demonstrating that the endogenous auxin within the cotyledon explant was adequate for callus stimulation with the absence of exogenous plant hormones. Besides, compact callus is green due to the green pigment of the chlorophyll, whereas yellow is lacking in chlorophyll (Figure 3). This current study is in agreement with Sari *et al*. (2018), whereby the color of callus is influenced by the plant growth regulator treatments in which the combination of auxin and cytokinin resulted in greener callus due to the presence of cytokinin compared to auxin alone. This report also indicated that the green color of the callus occurred due to the formation of chlorophyll stimulated by cytokinin in the presence of light, whereas the yellow callus lacked chlorophyll due to the chlorophyll degradation process or the lack of cytokinin. Additionally, according to Sari *et al*. (2018) and Luo *et al*. (2023), auxin inhibits chlorophyll formation in callus by suppressing the expression of genes involved in chlorophyll biosynthesis.

The effects of sucrose concentrations on friable callus induction for white and blue butterfly pea plants

In the current study, 15 g/L of sucrose was found to be the ideal concentration for inducing callus in both white and blue butterfly pea plants. The supplementation of 15 g/L of sucrose resulted in a high callus induction rate for both varieties with the values 87.5% (white variety) and 100% (blue variety) compared to the control, 0% (white and blue varieties). In the white variety, 1.0 mg/L of 2,4-D with 15 g/L sucrose was the optimal treatment. This concentration managed to yield the maximum fresh weight of

callus (0.146 ± 0.032 g) (Table 4). In Table 4, the results indicated that there were significant differences between the 1.0 mg/L of 2,4-D with 15 g/L sucrose with the rest of the treatments except for 0.75 mg/L of 2,4-D and 0.50 mg/L of 2,4-D with 15 g/L sucrose. However, the optimal treatment in the blue variety was 0.25 mg/L of 2,4-D with 15 g/L sucrose as it had the maximum fresh weight of callus (0.245 ± 0.075 g) (Table 5). This treatment is significant compared to the other with treatments. However, no significant difference was observed with the 0.5 mg/L of 2,4-D with 15 g/L sucrose. In addition, these current results are in agreement with the findings by Teoh *et al*. (2023), contradicting to the observation for the optimal treatment of the white variety. Hence, these findings revealed that the 2,4-D may produce callus induction at different levels of concentrations in both varieties. This outcome is consistent with a few studies in which 15 g/L of sucrose or lower sucrose concentrations was reported to be optimal for callus induction from leaf explants of *Aquilaria malaccensis, Vigna subterranea*, and *Paphiopedilum niveum* (Konate *et al*., 2013; Jayaraman *et al*., 2014; Chaireok *et al*., 2015).

Table 4. The effects of sucrose concentration on callus induction from cotyledon explants of white butterfly pea plants. *N*=90

Sucrose (g/L)	Treatments of 2,4-D (mg/L)	Callus Induction (%)	Fresh Weight (g)	Callus Morphology
15	0.00	0.0	0.000 ± 0.000^{d}	N/A
	0.25	100.0	0.089 ± 0.009^{bc}	Friable
	0.50	100.0	0.110 ± 0.011^{ab}	Friable
	0.75	87.5	0.143 ± 0.036^{a}	Friable
	1.00	87.5	0.146 ± 0.032^{a}	Friable
30	0.00	25.0	0.001 ± 0.001^{d}	Friable
	0.25	62.5	0.016 ± 0.006^{d}	Friable
	0.50	100.0	0.038 ± 0.004^{cd}	Friable
	0.75	100.0	0.039 ± 0.007^{cd}	Friable
	1.00	75.0	0.040 ± 0.012^{cd}	Friable

The data were presented as mean ± SE. Mean values followed by the same letter were not significantly different (Duncan test at $p \leq 0.05$)

Table 5. The effects of sucrose concentration on callus induction from cotyledon explants of blue butterfly pea plants. *N*=90

Sucrose (g/L)	Treatments of 2,4-D (mg/L)	Callus Induction (%)	Fresh Weight (g)	Callus Morphology
15	0.00	0.0	0.000 ± 0.000^{d}	N/A
	0.25	100.0	0.245 ± 0.075^{a}	Friable
	0.50	84.0	0.166 ± 0.058^{ab}	Friable
	0.75	87.5	0.121 ± 0.027^{bc}	Friable
	1.00	94.0	0.123 ± 0.039^{bc}	Friable
30	0.00	0.0	0.000 ± 0.000^{d}	N/A
	0.25	91.0	0.049 ± 0.016^{cd}	Friable
	0.50	91.0	0.021 ± 0.006^{cd}	Friable
	0.75	68.8	0.028 ± 0.009^{cd}	Friable
	1.00	78.0	0.043 ± 0.014^{cd}	Semi- Friable

The data were presented as mean ± SE. Mean values followed by the same letter were not significantly different (Duncan test at $p \leq 0.05$)

Sucrose is one of the carbon sources and a significant component for *in vitro* culture medium to fulfill the needs of *in vitro* plants as a limitation for regular photosynthesis activity, namely gas exchanges. Thus, carbohydrates from carbon sources allow the maintenance of the osmotic potential in the cells and stimulate the developmental process. Besides, the addition of exogenous sucrose is the main source of energy to the callus by increasing the cell respiration leads to increased cell mass (Wahyuni *et al*., 2020). Based on the Sari *et al*. (2018) study, this occurs when the osmolality of the medium is increased, and the fructose and glucose are formed via hydrolyzation of the sucrose leading to the diffusion of sucrose to the cell (osmosis), causing high turgor pressure. This turgor pressure aids in the magnification and elongation of the callus cells. Then, both glucose and fructose will undergo glycolysis and enter the Krebs cycle to produce energy (ATP) for the growth of the callus. However, if the sucrose in the medium is at high concentration, the sucrose will inhibit the photosynthesis process and no ATP is produced for the callus growth (Sari *et al*., 2018). Hence, in the treatment of 15 g/L sucrose, the callus formation is high as the turgor pressure in the cell is maintained. Furthermore, this study also reported that the inhibition of photosynthesis and chlorophyll formation occurred at high sucrose concentrations and caused the color variation of the callus. The type of callus can also be influenced by the concentration of sucrose, as Tajadod *et al*. (2012) documented on the color and morphology

changes at high concentrations of sucrose, such as yellow-deep color, dehydrated, rigid, and hard callus. On the other hand, high sucrose concentration also may cause the browning of the callus (Liang *et al*., 2019). These findings are aligned with the present study showing that callus treated with 15 g/L of sucrose for both varieties had yellowish-green or light yellowish color compared to the callus treated with 30 g/L of sucrose (Figures 4 & 5).

Fig. 4. Callus induction from cotyledon explants of white butterfly pea plants in half-strength MS medium supplemented with 2,4-D after 60 days. **(a)** Control (0 mg/L) + 15 g/L sucrose, **(b)** 2,4-D (1.0 mg/L) + 15 g/L sucrose, **(c)** Control (0 mg/L) + 30 g/L sucrose and **(d)** 2,4-D (1.0 mg/L) + 30 g/L sucrose. The scale bar represents 1 cm.

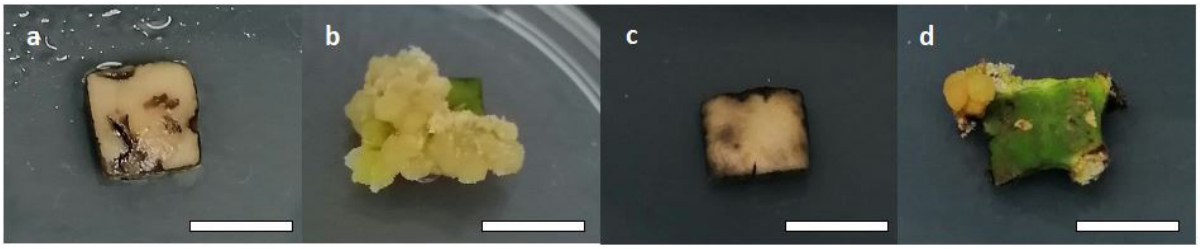

Fig. 5. Callus induction from cotyledon explants of blue butterfly pea plants in half-strength MS medium supplemented with 2,4-D after 60 days. **(a)** Control (0 mg/L) + 15 g/L sucrose, **(b)** 2,4-D (0.25 mg/L) + 15 g/L sucrose, **(c)** Control (0 mg/L) + 30 g/L sucrose, and **(d)** 2,4-D (0.25 mg/L) + 30 g/L sucrose. The scale bar represents 1 cm.

Sucrose is the primary carbon source commonly utilized in callus formation (Barreto Ferreira, 2018). In line, sucrose was the best choice of carbon source in callus induction of *Vigna subterranea, Phaseolus vulgaris,* and *Cannabis sativa* when subjected to a modified MS medium (Mello *et al*., 2001; Konate *et al*., 2013; Barreto Ferreira, 2018). The callus generated from cotyledon explants of white and blue butterfly pea plants treated with 15 g/L sucrose in this current study was friable, sticky, and appropriate for cell suspension cultures. As Ribeiro *et al*. (2021) stated, the consistency of friable callus is a significant component in establishing cell suspension culture as the soft and loose texture of the callus enables the homogenous dispersion of cells to the liquid nutrition medium. In addition, the importance of sucrose studies in secondary metabolites production was highlighted in *Chrysanthemum morifolium* (high quercetin content in 45 g/L sucrose) and *Mucuna pruriens* (high yield of L-DOPA in 2% of sucrose) (Rakesh & Praveen, 2022; Setiawati *et al*., 2023).

CONCLUSION

This study reports on the friable callus induction from cotyledon explants in two varieties of *C. ternatea,* white (*C. ternatea* var. Albiflora) and blue butterfly pea plants (*C. ternatea).* Optimal friable callus was induced in the combined treatments of 2,4-D and BAP for the white variety with the highest fresh weight induced in the treatment of 0.40 mg/L of 2,4-D and 0.50 mg/L of BAP. As for the blue variety, single 2,4-D treatments were observed to be more effective in friable callus induction with a concentration of 0.25 mg/L resulting in the highest callus fresh weight. This study also identified 15 g/L of sucrose as the optimal concentration for inducing friable callus in both varieties. The present study functions as a fundamental study for subsequent studies for establishing cell suspension cultures to harness novel secondary metabolites particularly pentacyclic triterpenoids associated with the mammalian neuroprotective properties.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL STATEMENT

Not applicable.

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