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Original Article

Effect of light and cytokinin on growth and curculin gene expression of *Curculigo latifolia* on in vitro culture

Efeito da luz e da citocinina no crescimento e na expressão do gene da curculina de *Curculigo latifolia* em cultura in vitro

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Abstract

Despite being valuable for producing a natural sweetener Curculin, *Curculigo latifolia* has a low growth and difficult to domestificate. So, to solve this problem, propagation on in vitro culture will be an alternative method to propagated this spesies under different cytokinins and light condition. Cytokinins and light has major role in organogenesis, growth and gene expression of many species. Thus, in this study, we aimed to improve the *Curculigo latifolia* growth on in vitro condition and expression of curculin gene by combining cytokinins addition and different light exposure. Four weeks seedlings were sub-cultured into medium (MS free hormone) containing 3 mg/L benzyladenine (BA) and various concentrations of meta-Topolin (mT) including 0.1 mg/L, 0.5 mg/L, and 5 mg/L. The cultures then incubated under different light types (red, blue, white LED lights and white fluorescence light) with 16-h light/ 18-h dark photoperiod for 14 weeks at 25 ± 2°C. Several parameters, including plant height, leaf number, chlorophyll contents, stomatal structure, and density and curculin expression, were observed every week. Unexpectedly, our results showed that *C. latifolia* growth displayed significant improvement when it was treated under white LED light without any additional cytokinins. In sum, white LED light further improves plantlets phenotype, such as plant height, leaf number, chlorophyll production, and stomatal number and structure, whereas, red LED light lead to a decreased phenotypes but increase the curculin gene expression.

Keywords: *Curculigo latifolia* growth, sweet-tasting protein-producing plants, LED light types, cytokinins. curculin gene.

Resumo

Apesar de ser valiosa para a produção do adoçante natural curculina, a *Curculigo latifolia* tem crescimento baixo e é de difícil aclimatação. Portanto, para resolver esse problema, a cultura in vitro é um método alternativo para propagar essa espécie em diferentes condições de luz e citocininas. As citocininas e a luz têm papel importante na organogênese, no crescimento e na expressão gênica de muitas espécies. Assim, neste estudo, nosso objetivo foi melhorar o crescimento de *C. latifolia* em condições in vitro e a expressão do gene da curculina, por meio da adição de citocininas e diferentes exposições à luz. As mudas de quatro semanas foram subcultivadas em meio (MS sem hormônio) contendo 3 mg/L de benziladenina (BA) e várias concentrações de metatopolina (mT), incluindo 0,1 mg/L, 0,5 mg/L e 5 mg/L. Em seguida, as culturas foram incubadas sob diferentes tipos de luz (luzes LED vermelha, azul, branca e luz de fluorescência branca) com fotoperíodo de 16 horas de luz por 18 horas de escuridão, por 14 semanas, a 25 ± 2°C. Vários parâmetros, incluindo altura da planta, número de folhas, conteúdo de clorofila, estrutura estomática, densidade e expressão de curculina, foram observados a cada semana. Inesperadamente, nossos resultados mostraram que o crescimento de *C. latifolia* apresentou melhora significativa quando em tratamento sob luz LED branca sem nenhuma citocinina adicional. Em suma, a luz LED branca melhora ainda mais o fenótipo das plântulas, como a altura da planta, o número de folhas, a produção de clorofila e o número e a estrutura dos estômatos, ao passo que a luz LED vermelha leva à diminuição do fenótipo, mas aumenta a expressão do gene da curculina.

Palavras-chave: crescimento de *Curculigo latifolia*, plantas produtoras de proteína de sabor doce, tipos de luz LED, citocininas, gene da curculina.

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1. Introduction

Curculigo latifolia (*Lemba* or *Marasi*) belongs to the family of *Hypoxidaceae*. It is a herbaceous plant with esteemed economic value due to its diverse benefits. However, propagation of this plant is not domestically well established (Raden et al., 2017). The growth rate of this plant is slim, making it difficult to be conventionally propagated. Previous study by Ismail et al. (2010) reported that the seed germination requires intensive care and special media. A considerable effort has been made by Farzinebrahimi et al. (2016) in propagating this plant through its rhizome, but it was failed. The promising method, so far, is in vitro propagation. Babaei et al. (2014) demonstrated micropropagation by using shoot tips of Malaysian Lemba. The explants were cultured in a basal medium containing various concentrations of thidiazuron (TDZ) ranging from 0 to 2 mg/L and indole-3-butyric acid (IBA) ranging from 0 to 0.5 mg/L. However, Farzinebrahimi et al. (2016) successed to grow callus under basal medium containing 3% sucrose that is solidified with 2.5 g/L Gelrite with addition of IBA and various concentrations of BA ranging from 0.5 to 4 mg/L.

Supplementary hormones in the medium play important roles in successful *C. latifolia* growth. Cytokinins, one of the plant hormones, trigger cellular changes which are important for cell decision including cell development and adaptive responses from abiotic to biotic environment. Aromatic cytokinins used in this study include benzyladenine (BA) and meta-Topolin (mT). mT isolated from poplar leaves has high activity. It plays an important role in delaying senescence, increasing photosynthetic pigments, and modulating the antioxidant enzyme activities (Amoo et al., 2015; Aremu et al., 2012). Thus, mT successfully improves the development of root and shoot in the in vitro propagation of numerous species such as *Pyrus communis* (Lotfi et al., 2020), *Opuntia stricta* Haw (Souza et al., 2019), *Tecoma stans* (Hussain et al., 2019), *Salvia viridis* (Grzegorczyk-Karolak et al., 2020), *Sesamum indicum* (Elayaraja et al., 2019), *Saccharum officinarum* (Souza et al., 2019), *Syzygium cumini* L. (Naaz et al., 2019), and *Musa* spp (Aremu et al., 2012).

Not only hormones, but environmental condition is also important for the in vitro culture of explant. For example, light affects not only photosynthesis but also plant morphology and development. Previous study reported that it induced gene expression (Chatelle et al., 2018). Despite the numerous accomplishments on the photoregulation of plant development, very little information is available on the specific effect of light quality provided by LED source regulating gene expression (Gupta, 2017). Moreover, light quantity, quality, and exposure duration regulate plant growth and development. Despite the low grow rate of *C. latifolia*, in our previous study, we successfully done in vitro germination (Muslihatin et al., 2022). Therefore, in this study, we aimed to improve the *Curculigo latifolia* growth on in vitro condition and expression of curculin gene by combining cytokinins addition and different light exposure.

2. Materials and methods

Time and places conducted study. This study was conducted on January 2022 – February 2023. This study conducted on Laboratory Bosciences and Plant technology and Biotechnology Laboratory, Departement Biology, Institut Teknologi Sepuluh Nopember, Indonesia and Institut of Tropical Deseases of Airlangga University, Indonesia.

Medium composition and growth condition. C. latifolia seeds were sterilized and germinated according to Muslihatin et al. (2023). Four seedlings were subcultured into medium (MS free hormone pH 5.8) containing 3 mg/L BA and various concentrations of mT including 0.1 mg/L, 0.5 mg/L, and 5 mg/L. The cultures then incubated under different light types including red (λ = 660-665 nm), blue (λ = 460-465 nm), white (6,000-6,500 K) LED lights and white fluorescence light (Philip™ TL 30W), with 16-h light/ 18-h dark photoperiod for 14 weeks at 25 ± 2°C. Several parameters, including plant height, leaf number, chlorophyll contents, stomatal structure, and density and curculin expression, were observed every week.

Measurement of chlorophyll content. In brief, per replication, 0.1 g (fresh weight) of leaves was macerated in 5 ml ice-cold acetone by using a mortar and pestle. Thereafter, the solution was filtered through Whatman No. 1 filter paper. The absorbance of the supernatant was measured at 645 and 662 nm using a UV-visible spectrophotometer against a blank (acetone). Pigment contents were determined and expressed in µg per g fresh weight using the formulae below (Equations 1, 2 and 3) according to Aremu et al. (2012):

$$Chl\ a\ \left(Ca\right)\ =\ 11.24A_{662} -\ 2.04A_{645} \tag{1}$$

$$Chl\ b\ \left(Cb\right)\ =\ 20.13A_{645} -\ 4.19A_{662} \tag{2}$$

$$Total\ Chl\ =\ 7.05A_{662} +\ 18.09A_{645} \tag{3}$$

Stomatal structure and density. Structure of stomata was analysed using Scanning Electron Microscope (SEM) (FEI Inspect S50) with 2,500 and 10,000 of magnification. Leaf discs were coated by Aurum Paladium (AuPd). Stomatal characteristics (pore aperture and pore length) were electronically measured. Stomatal number of the adaxial and abaxial surface were observed using light microscope Olympus CX21 and optilab advance, and upgraded with magnification of 100. Stomatal number was electronically measured using Raster 3 software. Stomatal density was observed per unit area (mm^2).

Plant material for molecular analysis. Leaves from 20 weeks seedling of *C. latifolia* cultured on MS0 medium under different light condition mentioned before being used in this experiment.

Primer Designing and reference genes. The full length complementary DNA (cds) of Curculin genes (Accession: AB181490.1) were used to query and were obtained from the publically available platform at NCBI. For curculin genes, primer pairs were designed using the online software (OligoAnalyzer, 2023).

Primers were choosed by parameters optimal length 20–25 nucleotides, melting temperature 60–65º C, GC content < 50%, product size range 100-446 base pairs, no self complementarities at 3' end, and absence for the hairpin structures and self-dimers. The qRT-PCR primer was designed with same steps (the primers were listed on Table 4). In this study, Ubiquitin was used as reference gene. The primer for Ubiquitin gene used primers according to Okubo et al. (2021).

RNA isolation and cDNA synthesis. The leaves (50 mg) were grinded. *Total RNA*TM *Mini Kit (Plant Geneaid*) was used to isolate total RNA according to the manufacturer's instructions. RNA quality was further assessed using the Nanodrop (Thermo Scientific™ Nanodrop 2000). The cDNA was synthesized from total RNA using GoScript™ Reverse Transcription System (Promega, USA) according to manufacturer's instructions. The reactions were placed in a controlled-temperature heat block equilibrated at 25°C, and incubate for 5 minutes, then incubated at 42°C for 60 minutes. The extension temperature may be optimized at 70°C for 15 minutes.

PCR Assay. 3 µL The cDNA sample was mixed with 25 µL NEXpro™ HS PCR 2X Master Mix, 5 µL *Nuclease Free Water*, 1 µL Primer Foward, 1 µL Primer Reverse (Primers are listed on Table 1). The reactions were placed in a thermal cycler (Select cycler II, Taiwan). This study used 35 cycle with preheated to 95°C for 7 minutes, denaturated to 95°C for 30 seconds, temperature of annelling 54°C for 30 seconds, temperature of extention 72°C for 40 seconds, and final extention 72°C for 7 minutes. Products PCR of cDNA were analyzed or detected using agarose gel electrophoresis

Electrophoresis. DNA visualization was performed using agarose gel electrophoresis technique. Preparation of 2% agarose gel was carried out by dissolving 2 g of agarose in 100 ml of 1X Tris Borate EDTA (TBE) solution and then heating in the microwave for 2 minutes. 20 µL/20 ml of gel dye RedSafeTM (IntRON Biotechnology) was added to the agarose solution (IntRON Biotechnology) and then left for 15 minutes until the solid agarose became a gel. Then the agarose gel was placed in an electrophoresis bath which contained 1x TBE solution until the gel was submerged. 5 µl The DNA sample was tested and 3 µL DNA leader 100 bp was added. Furthermore the electrophoresis was carried out for 30 minutes at 100V. The concentration and quality of electrophoretic RNA results were observed under UV transilluminator. The quality of the DNA is shown with a white line.

Real-time qRT-PCR Assays. Real-Time PCR was performed in real time PCR machine (MyGo pro, UK) with the fixed conditions (95°C for 120 seconds, 40 cycles of 15 s at 95°C for 15 second to 60°C 1 minute to 72°C) in final volume of 25 ml. The reactions involved GoTaq(R)qPCRMaster Mix) (Promega, USA), with composition 12,5 µL GoTaq (R) qPCR Master Mix, 5,5 µL *Nuclease Free Water,* 1 µL primer foward, 1 µL primer reverse and 5 µL cDNA. All reactions were run in three replicates, and Ubiquitin served as the endogenous reference gene.

Data analysis, this study used 200 seedlings. Each treatment, consisting of four light types and five variations of cytokinins concentration, was repeated 10 times. Data processing and analysis were performed using Microsoft Excel 2016 and SAS ver.25. The results were further statistically analyzed using two-way analysis of variance (ANOVA) followed by a Duncan Multiple Range Test (DMRT) at the significance level of 0.05.

3. Results

3.1. Different light types and supplementary cytokinins influence Morphology of C. latifolia

To observe the effect of light types on *C. latifolia* morphology, the plantlet was grown under different light types. The result showed that, except for red light, other light types exhibited not only higher *C. latifolia* plant, but also more leaves, with white fluorescence light being the best and followed by blue and white LED lights (Figure 1). Moreover, to know whether supplemental cytokinins (such as BA and mT) influence *C. latifolia* morphology, the plantlet was cultured in the medium containing BA or mT, as well as hormones-free medium. Surprisingly, *C. latifolia* plantlets, which were cultured in the hormones-free and mT-containing medium displayed higher plant height with more leaves. In contrast, the one cultured in 3 mg/L BA-containing medium exhibited lower plant height with less leaves (Figure 2). As shown in Figure 3, plantlets grown in a medium without hormones displayed normal morphology under any types of light. However, once they were grown in the medium with supplemental cytokinins, they exhibited distinct morphology. In the medium supplemented with 0.1 mg/L and 0.5 mg/L mT, plantlets grown under white fluorescence and blue LED light showed normal morphology, while the ones treated

Table 1. Effect of different light types and various cytokinins concentrations on *C. latifolia* height (cm).

Light	Cytokinins (mg/L)				
	MS0	**3 BA**	**5 mT**	**0.5 mT**	**0.1 mT**
Flouresence	2.85 ± 0.16 fg	1.21 ± 0.13 l	2.62 ± 0.17 h	2.11 ± 0.27 j	3.66 ± 0.15 c
White LED	5.17 ± 0.25 a	1.12 ± 0.13 l	3.25 ± 0.10 d	2.95 ± 0.16 ef	2.75 ± 0.08 gh
Blue LED	4.65 ± 0.16 b	0.81 ± 0.09 m	2.31 ± 0.13 i	3.08 ± 0.18 e	2.31 ± 0.11 i
Red LED	1.53 ± 0.20 k	1.05 ± 0.13 l	1.11 ± 0.26 l	0.88 ± 0.13 m	1.10 ± 0.23 l

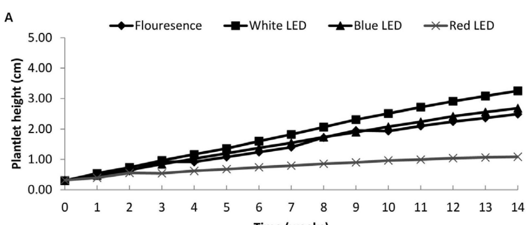
Note: numbers were followed by different letters show a significant difference based on the DMRT (α 0.05).

under white and red LED lights resulted in abnormal morphology with improper leaf development. Strikingly, in the medium containing ten-fold higher mT (5 mg/L), only plantlet grown in white LED light seemed to be normal, while others were abnormal. Moreover, the addition of 3 mg/L showed abnormal morphology. Overall, red LED light and supplemental cytokinins like mT and BA by up to 5 mg/L and 3 mg/L, respectively, impaired plantlets growth resulting in abnormal morphology (Figure 3).

To verify the effect of different light types and supplementary cytokinins, quantity analysis by using DMRT was carried out. The results showed these treatments significantly affect plant morphology. Consistent with the previous data, Tables 1 and 2 showed that plantlets, grown in the hormones-free medium, exhibited the highest plant height and leaf number (with the height of up to 5.17 cm and leaf number up to 6,5). In contrast, plantlets grown in the BA-containing medium possessed the lowest plant height and leaf number (with the height of only up to 0.81 cm and leaf number up to 1.0). Plantlets grown in the mT-containing medium resulted in average plant height. Moreover, we also tested the effect of light and supplementary cytokinins on the roots number (Table 3). Plantlets treated under red light showed no sign of root development in any medium, as well as plantlets in the BA-containing medium under any light types. The best root development was observed from plantlet grown under white LED light in the medium without any hormones.

3.2. Different light types and supplementary cytokinins significantly influence stomatal number but not stomatal structure of C. latifolia

To investigate whether different light types and supplementary cytokinins influence stomatal structure and number, stomata in both upper (adaxial) and lower (abaxial) parts of the leaf were observed. The results showed that, in general, stomatal number on the abaxial part was 5 to 12-fold higher than adaxial part under any lights treatment. To be more precise, in the hormones-free medium, the stomatal number was higher when plantlets were grown under white fluorescence and white LED light (with stomatal number reach about 115.49 unit/mm^2 and 118.98 unit/mm^2, respectively). In the 0.5 mg/L mT-containing medium, only plantlets grown under white LED light possessed the highest stomatal number up to 118.1 unit/mm^2. As for the ones in the 0.1 mg/L and



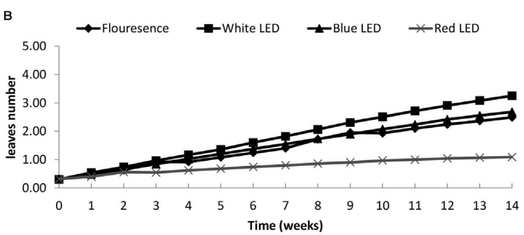
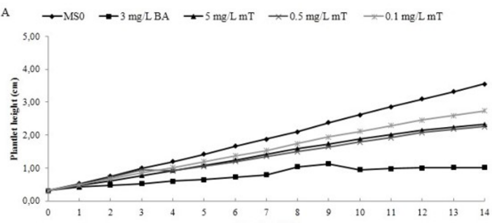


Figure 1. *C. latifolia* growth, treated under different light types, is observed through the (A) Plantlet height and (B) leaf number.



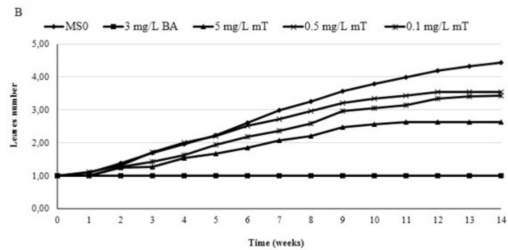


Figure 2. *C. latifolia* growth, cultivated in different medium, is observed through the (A) Plantlet height and (B) leaf number.

Table 2. Effect of different light types and various cytokinins concentrations on *C. latifolia* leaf number.

Light	Cytokinins (mg/L)				
	MS0	**3 BA**	**5 mT**	**0.5 mT**	**0.1 mT**
Flouresence	5.6 ± 0.51 b	1.0 ± 0 h	3.0 ± 0 ef	2.8 ± 0.42 f	5.6 ± 0.69 b
White LED	6.5 ± 0.84 a	1.0 ±. 0 h	3.3 ± 0.48 e	5.7 ± 0.48 b	4.0 ± 0.67 d
Blue LED	3.7 ± 0.54 d	1.0 ± 0 h	2.9 ± 0.31 ef	4.7 ± 0.48 c	3.1 ± 0.31 ef
Red LED	1.8 ± 0.42 g	1.0 ± 0 h	1.3 ± 0.48 h	1.0 ± 0.00 h	1.0 ± 0.00 h

Note: numbers were followed by different letters show a significant difference based on the DMRT (α 0.05).

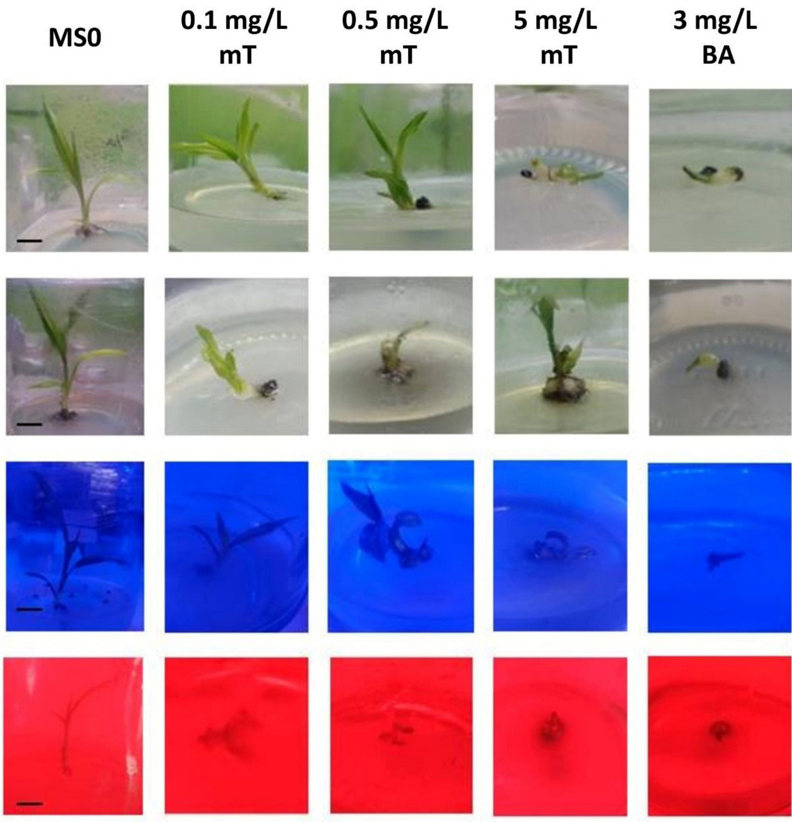


Figure 3. *C. latifolia* plantlets grown under different light types in the cytokinins-containing medium after 12 weeks of incubation.

Table 3. Effect of different light types and various cytokinins concentrations on *C. latifolia* root number.

Light	Cytokinins (mg/L)				
	MS0	**BA 3**	**mT 5**	**mT 0.5**	**mT 0.1**
Flouresence	3.6 ± 0.51 b	0.0 ± 0.0 e	1.4 ± 0.96 c	0.0 ± 0.0 e	1.1 ± 1.1 cd
White LED	**7.7 ± 1.41 a**	0.0 ± 0.0 e	1.1 ± 1.19 cd	0.6 ± 0.96 de	0 ± 0 e
Blue LED	3.6 ± 0.84 b	0.0 ± 0.0 e	0.0 ± 0.0 e	0.0 ± 0.0 e	0 ± 0 e
Red LED	0.0 ± 0.0 e	0.0 ± 0.0 e	0.0 ± 0.0 e	0.0 ± 0.0 e	0 ± 0 e

Note: numbers were followed by different letters show a significant difference based on the DMRT (α 0.05).

5 mg/L mT-containing medium, the highest stomatal number reach only up to 80 and 50 unit/mm^2 under white fluorescence light (Figure 4). Furthermore, according to the stomata structure depicted in Figure 5, although there were no abnormalities, plantlets grown under white fluorescence displayed closed stomata, while under other LED light types, the stomata were opened. There was no significance different on the stomata or pore size. The stomata size was 19.96 µm, 25.62 µm, 16.03 µm, and 25.44 µm for white fluorescence, white, blue, and red LED lights, respectively. The pore size of the opened stomata reached up to 13.61 µm, 13.38 µm, and 16.15 µm for white, blue, and red LED lights, respectively.

3.3. Different light types and supplementary cytokinins affect chlorophyll production

Chlorophyll is one of the indicators that demonstrate plant health. Therefore, we checked whether different light types and various cytokinins influence chlorophyll content. As shown in Figure 6, the results showed that the content of chlorophyll a was 2 to 3-fold higher than chlorophyll b under any types of light in any medium. While chlorophyll a is highly produced under white LED light, chlorophyll b is better under white fluorescence light. Interestingly, both chlorophyll a and b grown in the 0.1 mg/L mT-containing medium were highly produced under blue LED light. The highest chlorophyll a content

was produced by plantlets treated under white florescence in the hormones-free medium and blue LED light in the 0.1 mg/L containing medium, reaching up to 16,087 µg/g and 16,236 µg/g, respectively. The highest chlorophyll b content was obtained from platelets grown in the 0.5 mg/L mT-containing medium under blue LED light.

3.4. Different light types affect expression of curculin gene

The primers were used in the isolation and expression of the Curculin gene on the list (Table 4). PCR product were visualised by electrophoresis (Figure 7). From the visualization using agarose gel, it was showed that the gene encoding curculin was confirmed at 446 bp. Since qRT-PCR is relatively simple coupled with a high level of sensitivity, it is rapidly being adopted as a standard method for performing expression analysis of Curculin gene. From qRT-PCR result (Figure 8), it was showed that the expression was varied depend on the light. This study showed that LED light increased the expression level of Curculin gene. Especially, the gene expression was extremely inhibited under the flouresence light. However, apart from the control, red LED significantly promoted the expression level curculin as compared to the flouresence light. The curculin expression level of plants grown under blue LED was 1.79 fold higher and red LED was 2.43 fold higher than white LED light. Sedangkan gene expression under flouresence light was lower compare with under white LED.

4. Discussion

Taking all the data together, our result indicated that *C.latifolia* seedling displayed a normal and an optimum growth in the hormones-free medium compared to that of cytokinin-supplemented medium. Addition of BA and mT

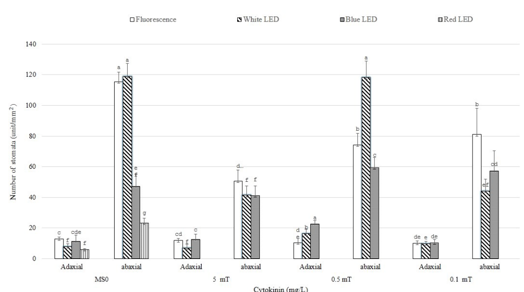


Figure 4. Effect of different light types and various concentrations of cytokinins on stomatal number in abaxial and adaxial parts of the leaf. Note: Numbers were followed by different letters show a significant difference based on the DMRT (α 0.05).

Table 4. Primer list for PCR and qRT PCR amplification.

Primer name	Sequences (primer direction 5'-3')
PCR	
Cur Fw	GCCAAGTTTCTTCTCACCATTC
Cur Rv	TCCTCATGTTGTGGTTCAGTAG
qRT PCR	
Cur Fw	GAGTGACGGGAACCTCATTATC
Cur Rv	CCATCCTGCTGAAGAACAAGA
Ubiquitin Fw*	TATAATCTGCAAGGGTCCGGC
Ubiquitin Rv*	AGATTCAGGACAAGGAGGGG

Note : * according to Okubo et al (2021).

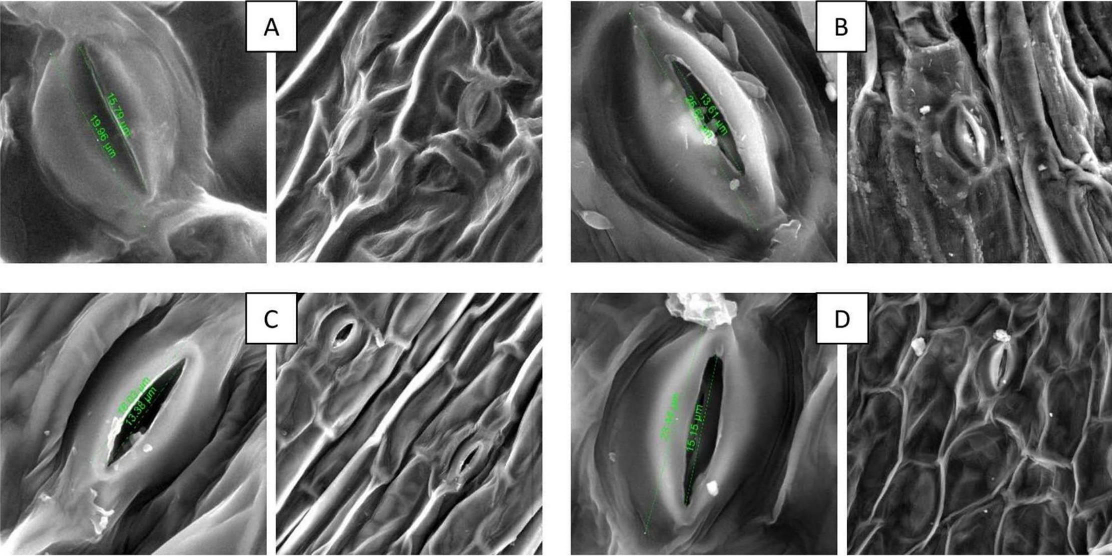


Figure 5. Morphology of *C. latifolia* stomata on MS0 medium under (A) flouresence, (B) white, (C) blue, and (D) Red LED lights observed by SEM with magnification of 10000 and 2500.

interfere the *C.latifolia* growth to some extent, including shoot and root development, chlorophyll production and stomatal opening. It is probably because BA can be metabolized to a toxic compound called N-glucosides or alanine conjugation. This compound is biologically inactive. Therefore, it is difficult to be hydrolyzed inside of the plant body, leading to a certain degree of accumulation (Werbrouck et al., 1996). In addition, (Bairu et al., 2011) reported that BA causes hyperhydricity in *Aloe polyphylla*. In contrat, previous study reported mT improves the development of root and shoot in the in vitro propagation of numerous species (de Souza et al., 2019; Elayaraja et al., 2019; Grzegorczyk-Karolak et al., 2020; Hussain et al., 2019; Lotfi et al., 2020; Naaz et al., 2019; Souza et al., 2019). Furthermore, previous studies reported that many species produces endogenous hormones which is sufficient for supporting its growth and development (Li et al., 2020; Raspor et al., 2020; Shreejana et al., 2023; Upadhyay et al., 2023; Yuan and Yang, 2018). Therefore, it is possible that extra hormones may have no effect or even trigger negative feedback as a response to excess number of hormones.

As for different light type treatments, our study reported that the growth of *C. latifolia* plantlets was majorly affected by white LED light. In line with our findings, Tran et al. (2018) reported that rice seedlings grown under white LED light displayed an optimum development. In addition, they reported that white and blue lights regulate chlorophyll production. White light promotes chlorophyll production in vanilla (Bello-Bello et al., 2016), lettuce (Kasim & Kasim, 2017), and sugarcane (Silva et al., 2020). Moreover, white light also contributes in synthesis of photosynthetic pigment (Song et al., 2022). Previous study reported that white light has broader spectral distribution, ranging from 400 to 700 nm. This spectrum easily reaches PAR region, allowing the plant to utilize the light (Burattini et al., 2017; Dugar et al., 2019; Tran and Jung, 2017). In contrast, other light types disturbed chlorophyll and carotenoid synthesis by reducing the activity of ALA, Proto IX, Mg-proto IX and Pchlide. Furthermore, our study reported that white LED light significantly enhances stomatal number.

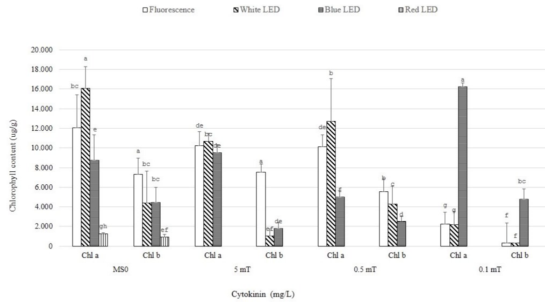


Figure 6. Effect of different light types and various concentrations of cytokinins on *C. latifolia* chlorofil a and b production. Note: Numbers were followed by different letters show a significant difference based on the DMRT (α 0.05).

Red LED has the opposite effect to white LED. The growth of *C. latifolia* is very low and has abnormal morphology under red LED irradiation. This result is in line with potato plantlets according to Chen et al. (2021), under red LED condition, *Solanum tuberisum* plantlets growth with small leaves, slim stems and weak roots. In contrast, red light exposure increased significantly the plant height of tomatoes, Arabidopsis, wheat, pepper, and other crops, the plant height under red light (Liang et al., 2022). Unfavour growth of *C latifolia* under red LED irradiation occurs because either PHYA or PHYB is sufficient for full responsiveness to red light**.** Reed et al. (1994), reported that PhyA can mediate inhibition of cell elongation and PhyB appears to play a major role in inhibition of hypocotyl elongation by red light in arabidopsis. However, red light slightly improves larger stomatal and pore size. Previous studies demonstrate that red light induces stomatal opening in *Gerbera jamesonii* (Meng et al., 2019). Moreover, red light promotes the accumulation of K^+ sugar via photosynthesis and starch degradation (Ando and Kinoshita, 2018) and induces PMH^+ ATPase activity in guard cells. Ion and sugar accumulation increase the water volume in the cell, leading to an increase in the size of the stomata (Inoue & Kinoshita, 2017).

The previous study documented that light has a pronounced effect on gene expression via photoreceptors particularly during the early photomorphogenetic development of plant (Li et al., 2017) . LED-regulated gene expression has been studied with respect to photoreceptors. Specifically, blue, red, and white LED, individually and/or in combination, regulate the expression of key regulatory genes involved in various metabolic pathways of plants (Gupta, 2017). But, the speculated that the response to light was species-specific. Even though the red light of the LED gave an unfavorable effect on the growth of *C. latifolia*, the red light caused the highest expression of the curculin gene. In *N. tabacum* red light providing activation of gene expression and in Arabidopsis, both PHYA and PHYB induction of gene expression in response to a brief red-light treatment (Müller et al., 2014; Reed et al., 1994). Subsequently, a red-light-controlled actuator, based on the N-terminal domains of PhyB and PIF6 from *Arabidopsis*

Figure 7. Confirmation of Curculin gene specific PCR amplification. M: marker, B: blue LED, F: flouresence, R: Red LED, W: white LED.

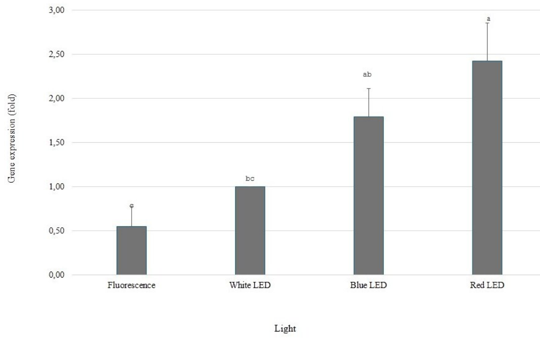


Figure 8. Relative expression of Curculin gene on different light condition.

thaliana, was demonstrated to achieve a high dynamic range of gene expression induction in *Nicotiana tabacum* and *Physcomitrium patens*-derived protoplasts in response to 660 nm red light (Larsen et al., 2023). In addition, red light also increases the gene expression level of strigolactone signal transduction genes SMXL, D14 and BRC2 in Pepino (*Solanum muricatum*) (Si et al., 2022). In addition, Zaghdoud et al. (2023), reported Red LED irradiation in seeds of Pepper (*Capsicum annuum* L.) caused an increase in the expression of TIP genes (tonoplast intrinsic proteins TIP1;4, Isoform TIP1;6, and TIP4;1) PIP2;3 (plasma membrane intrinsic proteins) and NIP1;1NOD26-like intrinsic proteins. In the other study, The red LED light has an effect on gene expression of synthesis carotenoid in the flavedo of citrus fruit (Ma et al., 2015). Red LED treatment up-regulated the expression of citPSY, citCRTISO, citLCYb2, citLCYe, and citVDE genes. Ochoa-Fernandez et al. (2020) has described a protocol for a light-inducible expression system that is activated by red light to control gene expression in leaf protoplasts of *Nicotiana tabacum* and *Arabidopsis thaliana*. genes, e.g. firefly luciferase. Upon exposure to red light, PhyB changes its conformation by photoisomerization of the covalently bound chromophore, phytochromobilin (PΦB). The activated form of PhyB (Pfr) binds to PIF6 and the VP16 domain is then recruited to the etr motif in close proximity to the minimal promoter, activating transcription of the reporter gene. In addition, Red light raises gene expression of BdCHS (Bradi4g17230) on a monocot model plant, *Brachypodium distachyon* (Tran et al., 2018).

5. Conclusion

There are several factors that influence the growth results in in vitro culture of targeted plants. In this study, composition and light media were studied, they provided an important role in the growth of *C. latifolia* in vitro. Both factors had a significant influence on the growth of *C. latifolia* on in vitro conditions. This study reported that hormones-free medium was sufficient to support *C. latifolia* growth on in vitro culture. According to the results obtained, not only growth, but light also influenced the expression of the Curculin gene of *C. latifolia.* The use of LED lighting as source of lighting in this research showed that LEDs were the smart choice for next-generation lighting source to improve growth of *C. latifolia* and increase of curculin gene expression. White LED light improved seedling phenotype, such as plant height, leaf number, chlorophyll production, and stomatal number and structure. It was found an interesting results from the experiments conducted that red LED light led to a decrease phenotype, but it increased the curculin gene expression. It was showed clearly that light used in the experiments had a significant effect to increase Curculin gene expression. By observing the expression of the Curculin gene obtained from experiments carried out, this study stated that the Curculin gene was an Inducible-light gene expression. From the results obtained, we found perspectives for the sustainability of this work and its applications. Inducible gene expression system is essential to control target gene expression with minimal or no interference for efficient large-scale Curculin and biopharmaceutical production. In the economic point of view, the growth of *C. latifolia* in hormone free medium shows that it is cost efficient for scale up propagation. The economic aspect is one of the critical aspects in biomass production that supports the realization of mass production of targeted product.

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References

AMOO, S.O., AREMU, A.O., MOYO, M., SUNMONU, T.O., PLÍHALOVÁ, L., DOLEŽAL, K. and VAN STADEN, J., 2015. Physiological and biochemical effects of a tetrahydropyranyl-substituted meta-topolin in micropropagated *Merwilla plumbea. Plant Cell, Tissue and Organ Culture*, vol. 121, no. 3, pp. 579-590. http://doi.org/10.1007/s11240-015-0728-0.

ANDO, E. and KINOSHITA, T., 2018. Red light-induced phosphorylation of plasma membrane H+-ATPase in stomatal guard cells. *Plant Physiology*, vol. 178, no. 2, pp. 838-849. http://doi.org/10.1104/pp.18.00544. PMid:30104254.

AREMU, A.O., BAIRU, M.W., SZÜČOVÁ, L., FINNIE, J.F. and VAN STADEN, J., 2012. The role of meta-topolins on the photosynthetic pigment profiles and foliar structures of micropropagated 'Williams' bananas. *Journal of Plant Physiology*, vol. 169, no. 15, pp. 1530-1541. http://doi.org/10.1016/j.jplph.2012.06.006. PMid:22883630.

BABAEI, N., ASHIKIN, N., ABDULLAH, P., SALEH, G. and ABDULLAH, T.L., 2014. An Efficient in vitro plantlet regeneration from shoot tip Cultures of *Curculigo latifolia*, a medicinal plant. *TheScientificWorldJournal*, vol. 2014, pp. 275028. http://doi.org/10.1155/2014/275028. PMid:24723799.

BAIRU, M.W., NOVÁK, O., DOLEŽAL, K. and VAN STADEN, J., 2011. Changes in endogenous cytokinin profiles in micropropagated *Harpagophytum procumbens* in relation to shoot-tip necrosis and cytokinin treatments. *Plant Growth Regulation*, vol. 63, no. 2, pp. 105-114. http://doi.org/10.1007/s10725-010-9558-6.

BELLO-BELLO, J.J., MARTÍNEZ-ESTRADA, E., CAAMAL-VELÁZQUEZ, J.H. and MORALES-RAMOS, V., 2016. Effect of LED light quality on in vitro shoot proliferation and growth of vanilla (*Vanilla planifolia* Andrews). *African Journal of Biotechnology*, vol. 15, no. 8, pp. 272-277. http://doi.org/10.5897/AJB2015.14662.

BURATTINI, C., MATTONI, B. and BISEGNA, F., 2017. The impact of spectral composition of white LEDs on Spinach (*Spinacia oleracea*) growth and development. *Energies*, vol. 10, no. 12, pp. 1383. http://doi.org/10.3390/en10091383.

CHATELLE, C., OCHOA-FERNANDEZ, R., ENGESSER, R., SCHNEIDER, N., BEYER, H.M., JONES, A.R., TIMMER, J., ZURBRIGGEN, M.D. and WEBER, W., 2018. A green-light-responsive system for the control of transgene expression in mammalian and plant cells. *ACS Synthetic Biology*, vol. 7, no. 5, pp. 1349-1358. http://doi.org/10.1021/acssynbio.7b00450.

CHEN, L., WANG, H., GONG, X., ZENG, Z., XUE, X. and HU, Y., 2021. Transcriptome analysis reveals effects of red and blue light-emitting diodes (LEDs) on the growth, chlorophyll fluorescence and endogenous plant hormones of potato (*Solanum tuberosum* L.) plantlets cultured in vitro. *Journal of Integrative Agriculture*, vol. 20, no. 11, pp. 2914-2931. http://doi.org/10.1016/S2095-3119(20)63393-7.

DE SOUZA, L.M., SILVA, M.M. A., HERCULANO, L., ULISSES, C. and CAMARA, T.R. 2019. Meta-topolin: an alternative for the prevention of oxidative stress in sugarcane micropropagation. Hoehnea, vol. 46, no. 3, pp. 0–4. https://doi.org/10.1590/2236-8906-107/2018

DUGAR, A.M., LIGHTING ARABIA DUBAI, R. and BURHANI, H., 2019. White LED Light sources-merging architectural and horticultural lighting applications within interior environments. *International Journal of Horticultural & Crop Science Research*, vol. 9, no. 2, pp. 83-93.

ELAYARAJA, D., SUBRAMANYAM, K., VASUDEVAN, V., SATHISH, S., KASTHURIRENGAN, S., GANAPATHI, A. and MANICKAVASAGAM, M., 2019. Meta-Topolin (mT) enhances the in vitro regeneration frequency of *Sesamum indicum* (L.). *Biocatalysis and Agricultural Biotechnology*, vol. 21, pp. 101320. http://doi.org/10.1016/j.bcab.2019.101320.

FARZINEBRAHIMI, R., MAT TAHA, R., RASHID, K.A., ALI AHMED, B., DANAEE, M. and ROZALI, S.E., 2016. Preliminary Screening of Antioxidant and Antibacterial Activities and Establishment of an Efficient Callus Induction in *Curculigo latifolia* Dryand (Lemba). *Evidence-Based Complementary and Alternative Medicine*, vol. 2016, pp. 6429652. http://doi.org/10.1155/2016/6429652. PMid:27298625.

GRZEGORCZYK-KAROLAK, I., HNATUSZKO-KONKA, K., ZARZYCKA, M. and KUŹMA, Ł., 2020. The stimulatory effect of purine-type cytokinins on proliferation and polyphenolic compound accumulation in shoot culture of *Salvia viridis. Biomolecules*, vol. 10, no. 2, pp. 1-15. http://doi.org/10.3390/biom10020178. PMid:31991557.

GUPTA, S.D., 2017. *Light emitting diodes for agriculture: smart lighting*. Singapore: Springer Nature, 334 p. http://doi.org/10.1007/978-981-10-5807-3.

HUSSAIN, S.A., AHMAD, N., ANIS, M. and ALATAR, A.A., 2019. Influence of meta-topolin on in vitro organogenesis in Tecoma stans L., assessment of genetic fidelity and phytochemical profiling of wild and regenerated plants. *Plant Cell, Tissue and Organ Culture*, vol. 138, no. 2, pp. 339-351. http://doi.org/10.1007/s11240-019-01631-5.

INOUE, S.I. and KINOSHITA, T., 2017. Blue light regulation of stomatal opening and the plasma membrane H+-ATPase. *Plant Physiology*, vol. 174, no. 2, pp. 531-538. http://doi.org/10.1104/pp.17.00166. PMid:28465463.

ISMAIL, M.F., ABDULLAH, P.N.A., SALEH, G. and ISMAIL, M., 2010. Anthesis and flower visitors in *Curculigo latifolia* Dryand. *Biology and Life Sciences*, vol. 1, no. 1, pp. 13-15.

KASIM, M.U. and KASIM, R., 2017. While continuous white LED lighting increases chlorophyll content (SPAD), green LED light reduces the infection rate of lettuce during storage and shelf-life conditions. *Journal of Food Processing and Preservation*, vol. 41, no. 6, pp. 1-7. http://doi.org/10.1111/jfpp.13266.

LARSEN, B., HOFMANN, R., CAMACHO, I.S., CLARKE, R.W., CLARK LAGARIAS, J., JONES, A.R. and JONES, A.M., 2023. Highlighter: an optogenetic system for high-resolution gene expression control in plants. *PLoS Biology*, vol. 21, no. 9, pp. e3002303. http://doi.org/10.1371/journal.pbio.3002303. PMid:37733664.

LI, C.X., XU, Z.G., DONG, R.Q., CHANG, S.X., WANG, L.Z., KHALIL-UR-REHMAN, M. and TAO, J.M., 2017. An RNA-seq analysis of grape plantlets grown in vitro reveals different responses to blue, green, red LED light, and white fluorescent light. *Frontiers in Plant Science*, vol. 8, pp. 78. http://doi.org/10.3389/fpls.2017.00078. PMid:28197159.

LI, X., FEI, R., CHEN, Z., FAN, C. and SUN, X., 2020. Plant hormonal changes and differential expression profiling reveal seed dormancy removal process in double dormant plant-herbaceous peony. *PLoS One*, vol. 15, no. 4, pp. e0231117. http://doi.org/10.1371/journal.pone.0231117. PMid:32240252.

LIANG, Y., COSSANI, C.M., SADRAS, V.O., YANG, Q. and WANG, Z., 2022. The interaction between nitrogen supply and light quality modulates plant growth and resource allocation. *Frontiers in Plant Science*, vol. 13, pp. 864090. http://doi.org/10.3389/fpls.2022.864090. PMid:35599862.

LOTFI, M., BAYOUDH, C., WERBROUCK, S. and MARS, M., 2020. Effects of meta–topolin derivatives and temporary immersion on hyperhydricity and in vitro shoot proliferation in Pyrus communis. *Plant Cell, Tissue and Organ Culture*, vol. 143, no. 3, pp. 499-505. http://doi.org/10.1007/s11240-020-01935-x.

MA, G., ZHANG, L., KATO, M., YAMAWAKI, K., KIRIIWA, Y., YAHATA, M., IKOMA, Y. and MATSUMOTO, H., 2015. Effect of the combination of ethylene and red LED light irradiation on carotenoid accumulation and carotenogenic gene expression in the flavedo of citrus fruit. *Postharvest Biology and Technology*, vol. 99, pp. 99-104. http://doi.org/10.1016/j.postharvbio.2014.08.002

MENG, X., WANG, Z., HE, S., SHI, L., SONG, Y., LOU, X. and HE, D., 2019. LED-supplied red and blue light alters the growth, antioxidant status, and photochemical potential of in vitro-grown *Gerbera jamesonii* plantlets. *Weonye Gwahag Gisulji*, vol. 37, no. 4, pp. 473-489. http://doi.org/10.7235/HORT.20190048.

MÜLLER, K., SIEGEL, D., RODRIGUEZ JAHNKE, F., GERRER, K., WEND, S., DECKER, E.L., RESKI, R., WEBER, W. and ZURBRIGGEN, M.D., 2014. A red light-controlled synthetic gene expression switch for plant systems. *Molecular BioSystems*, vol. 10, no. 7, pp. 1679-1688. http://doi.org/10.1039/C3MB70579J.

MUSLIHATIN, W., JADID, N., MANUHARA, Y.S.W., and WERBROUCK, S., 2023. Effect of light on seed germination and growth on *Curculigo latifolia*. *Acta Hortic*, vol. 1359, pp. 105-111. http://doi.org/10.17660/ActaHortic.2023.1359.12.

MUSLIHATIN, W., MANUHARA, Y.S.W. and WERBROUCK, S., 2022. Seed characteristics of *Curculigo latifolia* and its prospect to in vitro propagation. *IOP Conference Series. Earth and Environmental Science*, vol. 1115, no. 1, pp. 012053. http://doi.org/10.1088/1755-1315/1115/1/012053.

NAAZ, A., HUSSAIN, S.A., ANIS, M. and ALATAR, A.A., 2019. Meta-topolin improved micropropagation in *Syzygium cumini* and acclimatization to ex vitro conditions. *Biologia Plantarum*, vol. 63, no. 1, pp. 174-182. http://doi.org/10.32615/bp.2019.020.

OCHOA-FERNANDEZ, R., ABEL, N. B., WIELAND, F. G., SCHLEGEL, J., KOCH, L. A., MILLER, J. B., ENGESSER, R., GIURIANI, G., BRANDL, S. M., TIMMER, J., WEBER, W., OTT, T., SIMON, R. and ZURBRIGGEN, M. D., 2020. Optogenetic control of gene expression in plants in the presence of ambient white light. *Nature Methods,* vol. 17, no 7, pp. 717–725. https://doi.org/10.1038/s41592-020-0868-y.

OKUBO, S., TERAUCHI, K., OKADA, S., SAITO, Y., YAMAURA, T., MISAKA, T., NAKAJIMA, K.I., ABE, K. and ASAKURA, T., 2021. De novo transcriptome analysis and comparative expression profiling of genes associated with the taste-modifying protein neoculin in *Curculigo latifolia* and *Curculigo capitulata* fruits. *BMC Genomics*, vol. 22, pp. 347. http://doi.org/10.1186/s12864-021-07674-3. PMid:33985426.

OLIGOANALYSER ™ Tool, 2023 [viewed 2023 Feb 23]. *Integrated DNA Technologies* [software]. Available from: https://www.idtdna.com/pages/tools/oligoanalyzer

RADEN, I., NUGROHO, C.C. and SYAHRANI, S., 2017. Identification and characterization of morphological diversity of Lemba (*Curculigo latifolia*) in East Kalimantan, Indonesia. *Biodiversitas (Surakarta)*, vol. 18, no. 4, pp. 1367-1376. http://doi.org/10.13057/biodiv/d180412.

RASPOR, M., MOTYKA, V., NINKOVIĆ, S., DOBREV, P.I., MALBECK, J., ĆOSIĆ, T., CINGEL, A., SAVIĆ, J., TADIĆ, V. and DRAGIĆEVIĆ, I.C., 2020. Endogenous levels of cytokinins, indole-3-acetic acid and abscisic acid in in vitro grown potato: a contribution to potato hormonomic. *Scientific Reports*, vol. 10, no. 1, pp. 1-13. http://doi.org/10.1038/s41598-020-60412-9s.

REED, J.W., NAGATANI, A., ELICH, T.D., FAGAN, M. and CHORY, J., 1994. Phytochrome A and phytochrome B have overlapping but distinct functions in Arabidopsis development. *Plant Physiology*, vol. 104, no. 4, pp. 1139-1149. http://doi.org/10.1104/pp.104.4.1139. PMid:12232154.

SHREEJANA, K., POUDEL, A., OLI, D. and POKHREL, S., 2023. Role of endogenous hormones in germination and dormancy and gene action on hormones: a comprehensive review. *Food Science and Nutrition Technology*, vol. 8, no. 3, pp. 000309. http://doi.org/10.23880/fsnt-16000309.

SI, C., YANG, S., LOU, X., ZHANG, G. and ZHONG, Q., 2022. Effects of light spectrum on the morphophysiology and gene expression of lateral branching in Pepino (*Solanum muricatum*). *Frontiers in Plant Science*, vol. 13, pp. 1–12. https://doi.org/10.3389/fpls.2022.1012086.

SILVA, T.D., BATISTA, D.S., FORTINI, E.A., CASTRO, K.M., FELIPE, S.H.S., FERNANDES, A.M., SOUSA, R.M.J., CHAGAS, K., SILVA, J.V.S.D., CORREIA, L.N.F., FARIAS, L.M., LEITE, J.P.V., ROCHA, D.I. and OTONI, W.C., 2020. Blue and red light affects morphogenesis and 20-hydroxyecdisone content of in vitro *Pfaffia glomerata* accessions. *Journal of Photochemistry and Photobiology. B, Biology*, vol. 203, pp. 111761. http://doi.org/10.1016/j.jphotobiol.2019.111761. PMid:31896050.

SONG, Y., SHANG, W., WANG, Z., HE, S., SHI, L., SHEN, Y., LOU, X. and SUN, Y., 2022. Effects of different light-emitting diode qualities on the growth and photosynthetic characteristics of *Spathiphyllum floribundum. Canadian Journal of Plant Science*, vol. 102, no. 4, pp. 911-925. http://doi.org/10.1139/cjps-2022-0026.

SOUZA, L.M., RIBEIRO BARBOSA, M., ZÁRATE-SALAZAR, J.R., LOZANO-ISLA, F. and RANGEL CAMARA, T., 2019. Use of meta-Topolin, an unconventional cytokinin in the in vitro multiplication of *Opuntia stricta* Haw. *Biotecnología Vegetal*, vol. 19, no. 2, pp. 85-97.

TRAN, L.H. and JUNG, S., 2017. Effects of light-emitting diode irradiation on growth characteristics and regulation of porphyrin biosynthesis in rice seedlings. *International Journal of Molecular Sciences*, vol. 18, no. 3, pp. 641. http://doi.org/10.3390/ijms18030641. PMid:28300754.

TRAN, Q.-G., HAN, Y.-J., HWANG, O.-J., HOANG, Q.T.N. and KIM, J.-I., 2018. Exploring responses to light in the monocot model plant, *Brachypodium distachyon. Korean Journal of Plant Reources*, vol. 31, no. 5, pp. 522-530. http://doi.org/10.7732/kjpr.2018.31.5.522.

UPADHYAY, R.K., MOTYKA, V., POKORNA, E., DOBREV, P.I., LACEK, J., SHAO, J., LEWERS, K.S. and MATTOO, A.K., 2023. Comprehensive profiling of endogenous phytohormones and expression analysis of 1-aminocyclopropane-1-carboxylic acid synthase gene family during fruit development and ripening in octoploid strawberry (Fragaria× ananassa). *Plant Physiology and Biochemistry*, vol. 196, pp. 186-196. http://doi.org/10.1016/j.plaphy.2023.01.031. PMid:36724703.

WERBROUCK, S.P.O., STRNAD, M., VAN ONCKELEN, H.A. and DEBERGH, P.C., 1996. Meta-topolin, an alternative to benzyladenine in tissue culture? *Physiologia Plantarum*, vol. 98, no. 2, pp. 291-297. http://doi.org/10.1034/j.1399-3054.1996.980210.x.

YUAN, X.K. and YANG, Z.Q., 2018. The effect of endogenous hormones on plant morphology and fruit quality of tomato under difference between day and night temperature. *Horticultural Science (Prague)*, vol. 45, no. 3, pp. 131-138. http://doi.org/10.17221/7/2017-HORTSCI.

ZAGHDOUD, C., OLLIO, I., SOLANO, C.J., OCHOA, J., SUARDIAZ, J., FERNÁNDEZ, J.A. and MARTÍNEZ BALLESTA, M., 2023. Red LED Light Improves Pepper (*Capsicum annuum* L.) seed radicle emergence and growth through the modulation of aquaporins, hormone homeostasis, and metabolite remobilization. *International Journal of Molecular Sciences*, vol. 24, no. 5, pp. 4779. http://doi.org/10.3390/ijms24054779. PMid:36902208.