https://doi.org/10.48048/tis.2024.7218

Effects of Low-Dose Kinetin, 2,4-D and Monochromatic Light Conditions on Flavonoid Content in Callus Culture of *Dioscorea esculenta*

Noor Aini Habibah[1,*], Ari Yuniastuti[2], Nugrahaningsih Nugrahaning[2], Safitri Safitri[1] and Anisa Dewi Sri Puspitasari[1]

[1]*Plant Tissue Culture Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, Indonesia*
[2]*Biochemistry Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang, Indonesia*

([*]Corresponding author's e-mail: nooraini@mail.unnes.ac.id)

Received: 16 June 2023, Revised: 7 August 2023, Accepted: 10 August 2023, Published: 1 January 2024

Abstract

The callus culture of *Dioscorea esculenta* is a potential strategy to produce efficacious bioactive compounds in multiplied production. It can be carried out by applying plant growth regulators (PGR) and environmental modification, including light-emitting diodes (LEDs) exposure. This study aims to investigate the impact of PGR and the stimulatory effect of LEDs on flavonoid profiles in *D. esculenta* callus culture. The study was conducted using combination variations of 2,4-D (0.5 and 1 ppm), kinetin (0.5 and 1 ppm), and LEDs stimulatory conditions (light and dark). The results showed that flavonoids are the dominant compounds found in lesser yam cultures, which reached more than 78 % in all treatments-specifically, adding 0.5 ppm kinetin and 0.5 ppm 2,4-D to *D. esculenta* callus cultures significantly increased flavonoids production compared to single PGR treatment. This shows there is a synergistic activity induced by 2,4-D and kinetin. Furthermore, monochromatic light exposure also significantly affects flavonoid production. Analysis of 5 main flavonoids, isoflavones, flavanones, kaempferols, quercetin, and epigallocatechin, proved that monochromatic light significantly increased flavonoids production compared to callus cultures were kept in a growth room under 24 °C temperature in the dark condition. This study confirmed that equally combining 0.5 ppm of 2,4-D and 0.5 ppm of kinetin under monochromatic LEDs increases flavonoid production in *D. esculenta* callus cultures. Further research must be conducted to describe how the bioactive compound synthesis signaling pathway involving PGR and monochromatic LEDs improves plant quality in the pharmaceuticals' role.

Keywords: Flavonoids, *Dioscorea*, LED, Monochromatic light

Introduction

Dioscorea esculenta is a tuberous plant from the Dioscoreaceae family, which is developed as an alternative food source because of its high carbohydrate, fiber, protein, and mineral content [1,2]. Furthermore, other studies have also shown that *D. esculenta* tuber contains secondary metabolite compounds such as steroids, saponins, tannins, and coumarins [3]. Antioxidant activity analysis from *D. esculenta* tuber extract against 1,1-diphenyl-2-picrylhydrazil (DPPH) also showed an increase in scavenging activity as concentration enhanced, reaching 79.33 % for 1,000 mg/mL of the extract [4]. On the other hand, recent studies justify several specific bioactive compounds, such as water-soluble polysaccharides [5,6], dioscorin [7], diosgenin [8], confusarin, and nudol [9], which are beneficial for pharmaceuticals. Furthermore, *D. esculenta* is alleged to have high phenolic compounds, such as flavonoids, which have the substantial ability of antioxidant activity [10]. Previous studies show that *D. esculenta* tuber contains high flavonoid contents up to 12.4 ± 0.46 mg/100 g and alkaloids reaching 1.89 ± 0.02 mg/100 g [11]. These results emphasize the potential ability of *D. esculenta* tubers as a source of potential secondary metabolites for therapeutic agents, such as antioxidants, immunomodulators, prevention of metabolic diseases (hypercholesterolemia, dyslipidemia, diabetes, and obesity), inflammation, and cancer [12].

Flavonoid production in *D. esculenta* tuber callus culture can be enhanced faster and massively using plant growth regulators (PGR). Previous research showed that enriching medium with 0.5 ppm of 2,4-dichlorophenoxy acid (2,4-D) and 0.5 ppm of kinetin increases callus size and flavonoid contents of the *D.*

esculenta callus culture [13]. Conditioning of the cultural environment, such as light, is one of the absolute requirements in conducting callus culture. However, the effects of light, especially light-emitting diodes (LED), commonly used in plant culture maintenance, affect the growth and secondary metabolites production still needs further investigation, especially in *D. esculenta* callus culture.

Various studies have shown the combinations and concentrations effect of kinetin and 2,4-D in callus culture; however, the results vary depending on the plant species and organ or tissue origin [14,15]. In *Taxus brevifolia* Nutt., a combination of kinetin and 2,4-D showed a linear stimulating effect on callus growth [16]. Furthermore, applying exogenous kinetin increases the content of crucial phytohormones in tomatoes grown under salinity stress. It increases the number of isoflavones and reduces the negative impact of NaCl-induced salt stress [17]. Overall, the effects of kinetin and 2,4-D on culture growth are complex and may be influenced by light. Further research is needed to validate the impact and appropriate doses of kinetin and 2,4-D as PGR, especially in light-affected environments, to increase the biosynthesis of potential metabolites in *D. esculenta*. Moreover, this study aims to investigate the impact of PGR and the stimulatory effect of LEDs on flavonoid profiles in *D. esculenta* callus culture.

Methods

Explant sterilization

The study was an experimental design using *D. esculenta* tubers were local varieties obtained from community agriculture in Somagede Village, Banyumas Regency, Central Java, Indonesia. The tuber was clean-washed and sterilized through 2 stages to eliminate endophytic bacterial and fungal spores. First, the tubers were soaked using 0.15 % bactericidal aminoglycoside antibiotic (v:v), with active ingredients streptomycin sulfate 25 % and tetracycline trihydrate 20 %, mixed with 0.15 % fungicide (v:v) with active ingredients manganese ethylenebis (dithiocarbamate) (polymeric) 72 % and zinc ethylenebis (dithiocarbamate) 8 %. The soaking process was carried out for 1 h and then washed twice using sterile water. The second sterilization stage was conducted by soaking the tuber in a bleach solution containing 5.25 % sodium hypochlorite (NaClO). This study's tuber sterilization method followed the Habibah & Safitri method (Indonesian patent no IDS000005247, year 2022).

Media preparation

The growing medium was prepared using the full strength of Murashige and Skoog (MS) (Cat. No: M519, PhytotechLab; Kansas, USA). Culture media was made from 4.43 g MS media, then added with 25 g of sucrose, 0.1 g myoinositol, and 8 g agarose, then put into an Erlenmeyer flask and added distilled water until the volume reached 1 L. The media was mixed until homogeneous, and then kinetin stock solution and 2,4-D were added according to the treatment (**Table 1**). The media's acidity (pH) was measured using pH and adjusted until it reached 5.8. After the pH adjustment, the media mixture was heated over a water bath shaker until all the components were well dissolved and homogeneously. The medium was poured into sterile bottles and tightly closed. The press in culture bottles was sterilized in an autoclave with a temperature of 121 °C for 15 min.

Table 1 Comparison of plant growth regulators (PGR) in the *D. esculenta* callus culture treatment group.

Groups	Plant growth regulator concentration (ppm)	
	Kinetin	**2,4-D**
K1D1	0	0
K1D2	1	0
K2D1	0	1
K2D2	0.5	0.5

The media for each treatment was divided into 6 glass bottles with a volume of 25 mL as a repeat and then sterilized using an autoclave at 121 °C for 15 min.

Callus induction

Sterilized tubers were aseptically cut into 2×2×0.5 cm^3, then inoculated in the MS growth medium. Two explants implanted in each medium jar, with 6 jars containing explants in each group, were tightly closed and placed on a culture rack in a conditioned chamber with a temperature of 24 °C illuminated by

LEDs with light intensity reaching 1,000 lux. Inoculated explants from each group were divided into 2 equal pieces. As many as 3 jar bottles were covered using black trash bags until protected from light, while the rest were placed without plastic caps. The percentage and morphology of callus were monitored every day until day 90th.

Extraction

Secondary metabolites were extracted following a modified procedure from Hao *et al.* [18]. The callus culture was dried using an oven at 60 °C for 5 - 7 days, then ground into powder using a mortar and pestle [13]. Callus powder was sieved through a 45-mesh size and then used for secondary metabolites extraction using 5 mL of methanol containing 1 % HCl (v/v) and 5 mL HCl of 2 N added. Then, the solution is incubated at a temperature of 90 °C in thermal incubators. The extract solution is dried and re-suspended in methanol.

LC-MS analysis

The extract obtained was dissolved with methanol solvent to a concentration below 100 ppm until a homogeneous solution was obtained. It was then centrifuged at 8,000 rpm for 10 min to separate the pellet and supernatant. Then, the obtained supernatant was used for the protein precipitation stage. Two mL of supernatant extract were inserted into a centrifuge tube, and 3 mL of acetonitrile acidified with 0.2 % formic acid and centrifuged at 8,000 rpm for 30 s. Supernatants were used for purification processes with Solid Phase Extraction (SPE) and then continued for LCMS with specifications described in **Table 2**.

Table 2 Parameter and specification of LC-MS properties.

Parameters	Specification
Apparatus	Shimadzu LC-MS - 8040 LC-MS
Column	Column Shimadzu Shim Pack FC-ODS (2×150 mm^2, 3 μm)
Filter membrane	cellulose acetate 0.45 μm
Injection volume	1 μL
Capillary voltage	3.0 kV
Column Temperature	35 °C
Mobile phase mode	Isocratic
Flow rate	0.5 mL/min
Mobile phase	Methanol 90 % with water
MS-focused ion mode	Io type [M]+
Collison energy	5.0 V
Desolvation gas flow	60 mL/h
Desolvation temperature	350 °C,
Fragmentation method	Low energy CID
Ionization	ESI
Scanning speed	0.6 s/scan (m/z scan range: 10 - 1,000)
source temperature	100 °C
Run time	80 min

Preparation of standard solutions

The traditional stock solutions of epigallocatechin (0.874 mg/mL), flavanone (0.500 mg/mL), isoflavone (0.400 mg/mL), kaempferol (0.223 mg/mL), and quercetin (0.410 mg/mL), were prepared in methanol and mixed well until homogenous. The stock solution was stored in the dark condition at 4 °C until used. Working solutions were prepared by appropriate dilution and admixture of the stock solution.

Validation of the assay

External solvent calibration was performed by mixing 300 μl of the multi-analyte standard with 260 μl of dilution solvent (acetic acid/water/acetonitrile/1/79/20, v/v/v) for various secondary metabolites. Standard solutions were prepared by serial dilution using a mixture of acetic acid/water/acetonitrile (1/49.5/49.5, v/v/v), with dilution levels including 1:2.5, 1:10, 1:25, 1:100, 1:250, 1:1,000, 1:2,500 and 1:10,000. The standard solutions absorbance readings were used to validate the linearity of the response, the linear curve and the 1/x weighted using the MultiQuant™ 2.0.2 software (Sciex, Foster City, CA, USA). The limit of detection (LOD) and limit of quantification (LOQ) were determined for each target compound by serial dilution of the standard solution until the signal-to-noise ratio (S/N ratio) of each compound reached 3 and 10, respectively.

The instrument accuracy was reevaluated using specific solutions 268 ng/mL for epigallocatechin, 25 ng/mL for flavanone, 5.3 ng/mL for isoflavone, 11.5 ng/mL of kaempferol, and 123 ng/mL for quercetin, in 6 replicate injections under the optimal conditions. Regarding the method's precision, the intra-day accuracy was examined by analyzing 6 replicates of 3 samples in 1 day, while the inter-day precision was determined in 3 consecutive days.

Peak identification

Analyte identification refers to Atmospheric Pressure Ionization (API) and Electrospray Ionization (EI) which identify organic compounds because of the detection of primary protonated molecules and metal ions or solvents. The results of the LC-MS readings were elucidated in a chromatogram. According to the comparison of retention time, molecule parent, and product ions with standards, all the peaks of target compounds were unambiguously identified using Shimadzu's Peakintelligence^{TM} software. The retention time of epigallocatechin, flavanone, isoflavone, kaempferol, and quercetin is 23.71, 11.41, 10.52, 10.32 and 11.43 min, respectively.

Data analysis

Data of secondary metabolite compounds (%) and flavonoids (μg/mL) were statistically analyzed using a two-way ANOVA test with a confidence level of $\alpha = 95$ % and significance (*p*-value) of ≤ 0.050. All analyses were performed using SPSS v.23, and data presentation was performed using Ms. Excel 2019.

Results and discussion

Applying plant growth regulators such as kinetin and 2,4-D and stimulus such as lighting conditions may have varying effects on *Dioscorea* species. In this study, the combination of kinetin and 2,4-D triggers callus growth better than a PGR treatment in dark or light conditions. The increase was indicated by the callus weight, which was the combination of kinetin and 2,4-D. Based on the observation, it is bigger than the control on the last day of observance. In addition, monochrome LED exposure during the culture process produces a greener callus than in dark conditions (**Figure 1**).

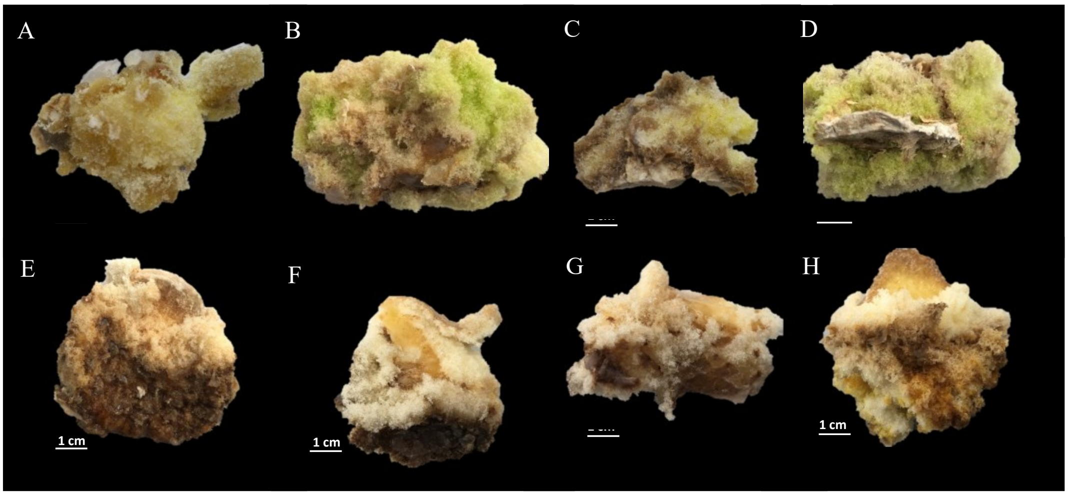

Figure 1 Morphology of *D. esculenta* callus produced in MS medium with growth factor supplementation. Respectively: A - D = K1D1; K1D2; K2D1; K2D2 in bright conditions and E - H = K1D1; K1D2; K2D1; K2D2 in dark conditions.

Several studies have also investigated the effects of kinetin, 2,4-D, and lighting conditions on the callus growth of some *Dioscorea* species. Kinetin significantly increased the growth and differentiation of callus *D. alata* propagated *in vitro* with an optimal concentration of 2.5 mg/L [19]. Furthermore, 2,4-D acts like auxin, significantly affecting plant calluses' growth, such as *Stelechocarpus burahol* [20] and *Elaeocarpus grandiflorus* [21]. However, the effect of the 2,4-D mechanism on *D. esculenta* callus growth remains unclear. In addition, instead of PGR, environmental factors during the culture process, especially light exposure, may increase the content of secondary metabolites. The current study also explains significant physiological changes in plants after light exposure treatment. Studies conducted on *D. zingiberensis* plants, which were maintained with a light intensity of 270 mE/m^{2}s changed grana structure in chloroplasts, escalated carotenoid and fatty acid content that increased leaf dry weight, and reduced diosgenin content in rhizomes [22].

In a study on the callus *of Durio zibethinus* Murr, using kinetin and 2,4-D produces a synergistic effect in increasing the growth of callus lignin content per cell [23]. In the culture study of *Dioscorea esculenta* L., using kinetin combined with light treatment significantly increased the percentage of culture biomass [24]. In another study, using kinetin greatly affected the growth of the tip of the propagate *shoot of D. trifida* L., with an optimal concentration of 0.5 mg/L [25, 26]. In addition, kinetin also significantly increases the phytochemical content in callus cultures *of Dioscorea bulbifera* L. The results are relevant to this study, where kinetin under light conditions resulted in the growth of *D. esculenta* callus culture faster than 2,4-D at the same dose and conditions. However, the combination of kinetin and 2,4-D at a low dose of 0.5 mg/mL produced the greatest growth compared to other treatments.

In this study, the LC-MS analysis of *D. esculenta* callus culture shows no significant effect of light and PGR on the secondary metabolites percentage (**Table 3**). It means the composition of the compounds is relatively the same in all treatments. However, the callus cultures in the dark showed more flavonoids than in light conditions, despite no significant difference.

Table 3 The concentration of secondary metabolites by class of compounds.

Compound groups	**Compound concentration (%)**							
	Light				**Dark**			
	K1D1	**K1D2**	**K2D1**	**K2D2**	**K1D1**	**K1D2**	**K2D1**	**K2D2**
Benzenoids	2.58	3.38	3.40	3.44	3.38	3.42	3.46	3.23
Carboxylic Acids	0.73	0.73	0.73	0.76	0.78	0.74	0.77	0.76
Carotenoids	1.14	0.57	1.01	0.60	0.41	0.84	0.60	0.33
Phenylpropanoids	5.19	5.50	4.82	5.59	5.72	5.26	5.61	5.48
Diarylheptanoids	1.85	0.34	1.16	0.21	0.54	0.53	0.55	0.69
Fatty Acyls	0.20	0.40	0.40	0.42	0.43	0.40	0.42	0.42
Flavonoids	79.36	79.80	79.67	80.41	81.36	80.70	81.63	80.23
Glucosides	0.47	0.48	0.48	0.50	0.51	0.48	0.50	0.50
Carbonyl	2.77	2.21	2.22	1.75	0.71	1.74	1.76	1.75
Phytosteroles	2.03	2.24	2.26	2.35	2.40	2.27	2.36	2.35
Stilbenoids	0.32	-	0.24	0.28	0.28	0.19	0.19	0.18
Terpenoids	1.64	1.39	1.59	1.62	1.57	1.29	1.27	1.54
Xanthonoids	2.60	2.97	2.99	2.18	2.59	2.45	2.01	2.53

Note: The statistical results show no significant difference in all compound concentrations (p-value > 0.050). The analysis was obtained using two-way ANOVA with a confidence level of α = 95 % and significance (p-value) ≤ 0.050.

Furthermore, several compounds' percentages were higher in callus cultures grown in bright conditions, such as carotenoids and carbonyl groups. It may refer to the chromatophore characteristic of carotenoids that requires light to be active or metabolized [27]. The flavonoid compounds were also measured, reaching more than 79.00 % in both growth conditions, making it the most dominant compound in the *D. esculenta* callus culture. This shows that the *D. esculenta* callus culture method has high potential in producing flavonoids massively. About 74 - 78 flavonoid compounds out of 117 secondary metabolites were identified from *D. esculenta* callus cultures, making this group the most abundant compared to other compounds (**Table 4**).

Table 4 Number of secondary metabolites by compound class.

Compounds groups	**Treatments**							
	Light				**Dark**			
	K1D1	**K1D2**	**K2D1**	**K2D2**	**K1D1**	**K1D2**	**K2D1**	**K2D2**
Benzenoid	3	3	3	3	3	3	3	3
Carboxylic Acid	2	2	2	2	2	2	2	2
Carotenoid	3	2	2	2	2	3	2	1
Phenylpropanoids	7	8	6	8	8	6	8	8
Diarylheptanoids	3	1	3	1	3	2	2	3
Fatty Acyls	1	1	1	1	1	1	1	1
Flavonoid	76	76	77	75	75	76	75	74
Glucoside	1	1	1	1	1	1	1	1
Carbonyl	4	4	4	3	2	3	3	3
Phytosterols	4	4	4	4	4	4	4	4
Stilbenoid	1	-	1	2	2	1	1	2
Terpenoid	4	4	4	5	5	4	4	5
Xanthonoid	3	3	3	2	2	3	3	3
Total compound*	112	109	111	109	110	109	109	110

Note: Differences in the total indicate the absence of certain compounds in some callus treatments

The various compound from the callus in different PGR and light conditions indicates several compounds that were not detected. The absence of some compounds is still enigmatic, although callus culture in dark conditions seems to produce a lower percentage compared with the light condition. Specific compounds such as 2.5-dimethoxy-p-benzoquinone from the carbonyl group were also not found in all calluses maintained in the dark (**Figure 2**).

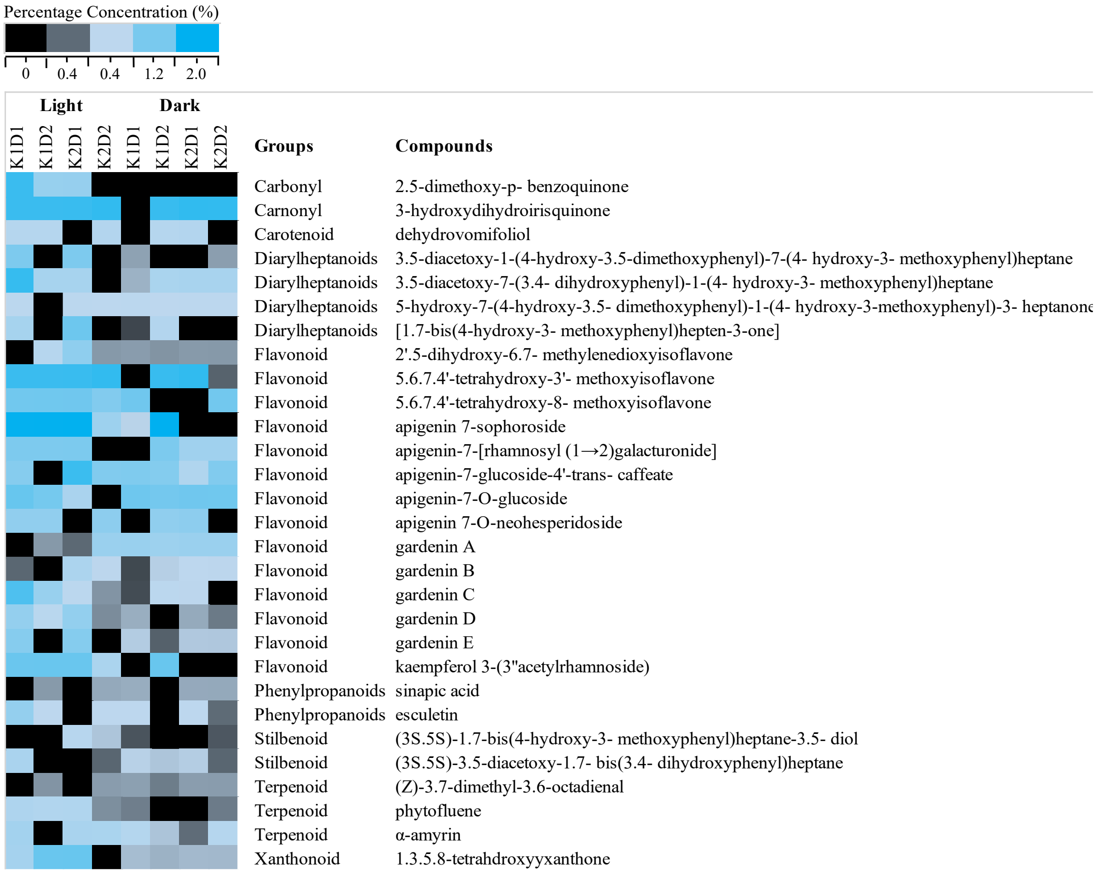

Figure 2 Specific compounds are absent in the callus culture treatment group. The black color represents the compound that is not found.

Table 5 The amount and average levels of major flavonoid compounds.

Flavonoids group	Compound members	Total concentration (%)							
		Light				Dark			
		K1D1	K1D2	K2D1	K2D2	K1D1	K1D2	K2D1	K2D2
Flavanone[4]	7	6.23	6.24	6.29	6.33	6.69	6.32	6.57	6.54
Apigenin	8	5.49	5.83	5.58	3.37	3.92	5.48	4.03	4.02
Catechin	1	1.18	1.18	1.19	1.24	1.27	1.20	1.25	1.24
Epiafzelechin	2	1.69	1.70	1.71	1.78	1.82	1.72	1.79	1.78
Epicatechin	1	0.87	1.34	1.35	1.41	1.44	1.36	1.42	1.41
Epigallocatechin[5]	4	5.73	5.74	5.78	6.02	6.16	5.81	6.05	6.02
Gardenin	5	1.99	0.80	1.64	0.89	0.93	0.91	1.17	0.92
Iriflogenin	1	1.10	1.19	0.60	1.25	1.28	1.20	1.25	1.25
Isoflavone[2]	13	12.20	12.87	11.89	12.79	12.10	11.89	12.25	11.91
Isorhamnetin	2	1.89	1.89	1.91	1.99	2.03	1.92	1.99	1.98
Kaempferol[1]	12	15.45	16.09	16.32	15.88	16.14	16.40	16.30	16.23
Luteolin	3	3.37	3.95	3.98	4.14	4.24	4.00	4.16	4.14
Narcissoside	1	1.06	1.07	1.07	1.12	1.14	1.08	1.12	1.12
Naringenin	1	1.34	1.34	1.35	1.41	1.44	1.36	1.42	1.41
Naringin	1	0.66	0.66	0.67	0.70	0.71	0.67	0.70	0.70
Nicotiflorin	1	1.44	1.44	0.72	1.51	1.55	1.46	1.52	1.51

Flavonoids group	**Compound members**	**Total concentration (%)**							
		Light				**Dark**			
		K1D1	**K1D2**	**K2D1**	**K2D2**	**K1D1**	**K1D2**	**K2D1**	**K2D2**
Pratensein	1	1.08	1.08	1.09	1.13	1.16	1.09	1.14	1.13
Quercetin[3]	5	8.01	6.20	8.10	8.43	8.62	8.14	8.46	8.42
Rhamnetin	1	0.34	0.34	0.34	0.36	0.36	0.34	0.36	0.36
Rutin	2	3.21	3.22	3.24	3.38	3.46	3.26	3.39	3.37
Scutellarein	1	1.44	1.45	1.46	1.52	1.55	1.46	1.52	1.52
Tectorigenin	2	1.04	1.04	1.28	1.09	1.12	1.05	1.09	1.09
Unidentified*	3	2.55	3.12	2.09	2.68	2.23	2.58	2.69	2.18

Description: The total concentration was performed by summarizing the concentration from all members in the same flavonoid group. Superscript numbers ($^{1-5}$) indicate the order of concentration (%) of flavonoid compounds based on the conditioning group and the effect of PGR. The star mark (*) represents the flavonoid compounds that cannot be classified into main flavonoid groups

In this study, from a total of 78 flavonoids, 5 main compound groups with the most significant percentage were found, there are kaempferol group (12 compounds); isoflavones (13 compounds); quercetin (5 compounds); flavanone (7 compounds); and epigallocatechin (4 compounds) as presented in **Table 5**. For further study, the quantitative analysis was focused on the members in each group that accordance with the standard stock solution for epigallocatechin, flavanone, isoflavone, kaempferol and quercetin in specific retention time. The analysis was conducted to obtain a main concentration of each flavonoid per dry weight of *D. esculenta* callus culture.

The statistical analysis shows no significant difference between the kinetin or 2,4-D effect on the production of flavonoid compounds in *D. esculenta* callus cultures under all conditions. However, all flavonoids increase by at least 30 % when combined at a low concentration. It shows that kinetin and 2,4-D may be involved in a synergetic cascade to stimulate the optimal biosynthesis of epigallocatechin, flavanone, isoflavone, kaempferol, and quercetin in *D. esculenta* callus culture (**Table 6**).

Table 6 Calculated levels of 5 dominant flavonoid compounds based on PGR conditioning and treatment.

Flavonoids	**Cond.**	**Concentration (µg/mL)**			
		K1D1	**K1D2**	**K2D1**	**K2D2**
Epigallocatechin	Light	$62.83 \pm 2.56^{a\dagger}$	$83.94 \pm 2.44^{b\dagger}$	87.97 ± 1.75^{b}	$113.77 \pm 1.45^{c\dagger}$
	Dark	$53.73 \pm 4.74^{a\ddagger}$	$78.92 \pm 2.84^{b\ddagger}$	85.45 ± 3.39^{b}	$95.35 \pm 4.38^{c\ddagger}$
Flavanone	Light	$366.20 \pm 27.60^{a\dagger}$	$463.20 \pm 42.60^{b\dagger}$	$495.15 \pm 5.62^{b\dagger}$	$663.15 \pm 25.53^{c\dagger}$
	Dark	$320.61 \pm 7.47^{a\ddagger}$	$435.45 \pm 38.79^{b\ddagger}$	$457.95 \pm 29.94^{b\ddagger}$	$555.74 \pm 8.47^{c\ddagger}$
Isoflavone	Light	$429.44 \pm 32.37^{a\dagger}$	$573.73 \pm 19.42^{b\dagger}$	547.25 ± 15.70^{b}	$658.84 \pm 26.36^{c\dagger}$
	Dark	$382.22 \pm 2.51^{a\ddagger}$	$493.10 \pm 22.77^{b\ddagger}$	565.64 ± 50.85^{bc}	$559.96 \pm 0.56^{c\ddagger}$
Kaempferol	Light	$137.62 \pm 10.36^{a\dagger}$	$183.81 \pm 6.22^{b\dagger}$	$192.63 \pm 7.41^{b\dagger}$	$249.11 \pm 9.58^{c\dagger}$
	Dark	$114.37 \pm 2.66^{a\ddagger}$	$172.84 \pm 5.35^{b\ddagger}$	$187.13 \pm 3.82^{c\ddagger}$	$208.78 \pm 3.18^{d\ddagger}$
Quercetin	Light	$125.33 \pm 9.44^{a\dagger}$	167.39 ± 5.66^{b}	175.42 ± 6.75^{b}	$226.85 \pm 8.73^{c\dagger}$
	Dark	$109.75 \pm 2.55^{a\ddagger}$	157.40 ± 4.87^{b}	170.41 ± 3.48^{c}	$190.13 \pm 2.90^{d\ddagger}$

Description: The letters of the superscript alphabet (a - d) indicate a significant difference in compound content due to the influence of PGR. Dagger marks ($^{\dagger,\ \ddagger}$) indicate a difference caused by the influence of light (dark-light). The statistical results were obtained using two-way ANOVA with a confidence level of $\alpha = 95$ % and significance (p-value) ≤ 0.050.

The analysis showed that the dark or light conditioning and the application of PGR significantly affect the flavonoid concentration (**Table 6**). Specifically, the monochromatic LEDs significantly trigger most of the flavonoid production compared to the dark condition. The standardized quantification levels show that flavanone concentration is the highest flavonoid compound produced in *D. esculenta,* followed by isoflavones, kaempferol, quercetin, and the last is epigallocatechin. It is inversely proportional to the number of combinations that isoflavones and kaempferol are the largest family groups of flavonoids.

The highest content of these 5 flavonoids (**Table 4**) is found in K2D2 callus culture and significantly differed from other groups. Furthermore, flavonoid production in K1D2 and K2D1 cultures is identified as relatively the same and not significantly different, but higher and substantially different from K1D1 cultures. The highest increased concentration of flavonoids in the *D. esculenta* callus culture was found under the monochromatic light conditions and significantly different from the callus culture in the dark conditions. Previous studies have also shown a significant increase in the biosynthesis of secondary metabolite compounds in callus cultures and calluses due to irradiation. Irradiating the callus of *Rhodiola imbricata* using LEDs significantly increased flavonoid synthesis [28]. In addition, significant increases in flavonoid compounds were also reported in *Eclipta alba* callus [29] and callus cultures [30]. The previous evidence is relevant to this study, where *D. esculenta* callus cultures exposed to light produced higher flavonoid levels than in dark conditions (**Figure 3**).

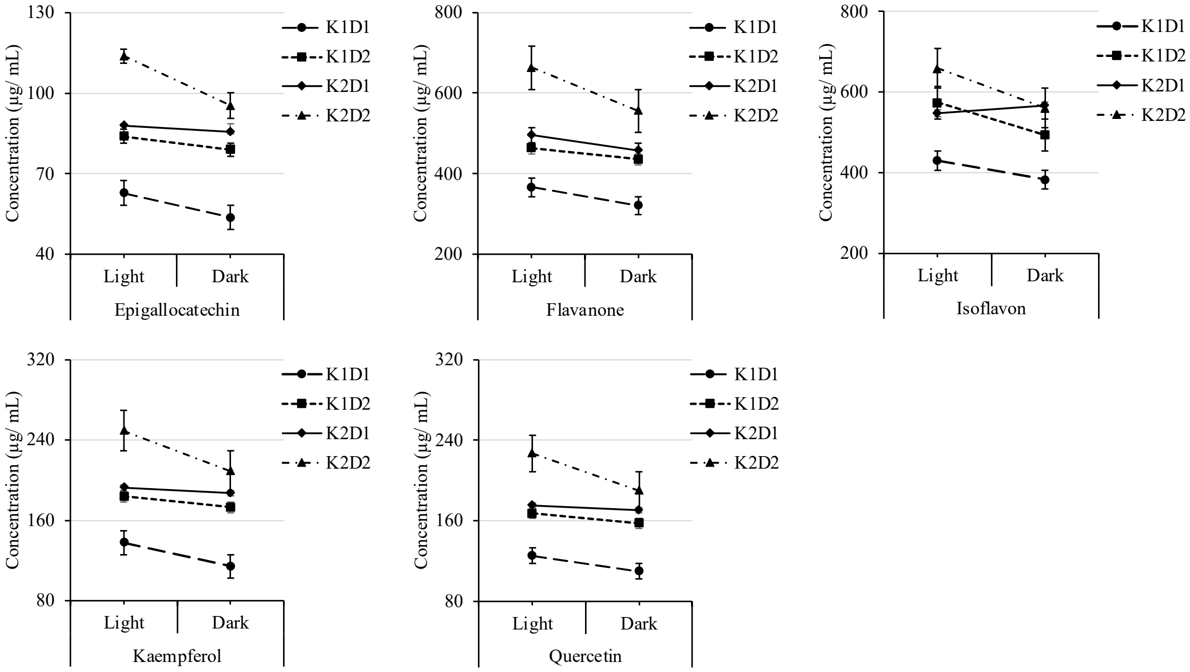

Figure 3 Graph of changes in flavonoid compound content in each PGR treatment group with conditioning in the form of lighting differences.

This study also showed that applying 0.5 mg kinetin or 2,4-D to *D. esculenta* callus culture separately did not significantly differ in the major flavonoid concentrations, except isoflavones. The results show a difference in the isoflavone compound of *D. esculenta* callus culture K2D1, where the levels are higher in dark conditions and significantly differ from K1D1 and K2D2. Furthermore, epigallocatechin, flavanone, kaempferol, and quercetin in dark conditions is lower than in light condition (**Table 6**).

Table 6 Changes in flavonoids levels of *D. esculenta* callus culture in light and dark conditions.

Flavonoids	Change in concentration (%)			
	K1D1	K1D2	K2D1	K2D2
Epigallocatechin	−14.49	−5.97	−2.86	−16.20
Flavanone	−12.45	−5.99	−7.51	−16.20
Isoflavone	−10.10	−14.05	+3.36	−15.01
Kaempferol	−16.89	−5.97	−2.86	−16.19
Quercetin	−12.44	−5.97	−2.86	−16.19

Description: The negative sign (−) indicates a greater concentration of flavonoids in *D. esculenta* callus culture in light conditions, while the positive sign (+) in *D. esculenta* callus culture in dark conditions.

The flavonoid concentration changes were observed most dramatically in kaempferol, where the decrease level reached more than 16 % in K1D1 and K2D2 cultures. In the dark condition, all flavonoid compounds in K2D2 callus cultures are decreased, ranging from 2 - 16 %. These results indicate that light plays an essential role in flavonoid biosynthesis. Several studies in different species have shown that light conditions induce flavonoid production [31,32] and antioxidant activity [33] higher than dark conditions. Dark conditions are assumed to be the most favorable conditions for *D. esculenta* callus growth. Furthermore, the concentration and composition of PGR approved affect the concentration of secondary metabolites and the antioxidant activity of *D. esculenta* callus [24]. It is relevant to this study, where *D. esculenta* callus culture was most optimally induced when it was maintained at a combination of 0.5 ppm kinetin and 0.5 ppm 2.4-D under light conditions. The interaction between kinetin, 2,4-D, and light in flavonoid biosynthesis showed synergistic effects, although the specific mechanism varied depending on plant species and experimental conditions. Crosstalk phytohormones likely perform a synergistic function by regulating gene expression of enzymes involved in regulating the biosynthesis of secondary metabolites, including flavonoids [34-36].

Kinetin, a cytokinin group, is a transcriptional activator in gene regulation related to cell differentiation and faces stress [37]. We assume dark conditions may trigger stress, probably silencing or activating several genes involved in metabolite seconder biosynthesis. In molecular mechanism. Kinetinis captured by cytokinin *Arabidopsis* histidine kinase-2 (AHK2), AHK3, and cytokinin response 1 (CRE1)/AHK4, which then activates multistep autophosphorylation of the amino acid histidine of the protein receptors in the cell membrane. All 3 proteins have cyclase/His kinase-associated extracellular sensing (CHASE), which binds to extracellular cytokinin [38]. Then the phosphorylation signal is forwarded up to the phosphoryl histidine group (His) and transferred via aspartate residues (Asp) in the cytoplasm [39]. CRE1/AHK4 that is not bound to cytokinin mediates the reverse phosphorus (P) group transfer reaction to dephosphorylate *Arabidopsis* His phosphotransfer protein (AHP). It is then translocated to the nucleus to the P groups of A-type *Arabidopsis* response regulators (ARR) or B-type ARR receiving domains on conserved Asp residues [40]. B-type ARR phosphorylation releases the repressor, thereby triggering the activation of transcription factors, including A-type ARR and cytokinin Response Factors (CRF). CRF protein is also activated by cytokinin via AHP to accumulate in the nucleus and activate the transcription of functional genes that play a role in circadian rhythms and phytochrome function [41]. In other words, the increase in kinetin leads to the rise in phytochrome function, which plays a role in light response to growth and dealing with stress.

Furthermore, the 2,4-D is a member of synthetic auxins that play an optimal role at low concentrations and justify activating secondary metabolite biosynthesis-related genes. 2,4-D behaves like auxin triggering the synthesis of the Aux/IAA complex with auxin response factor (ARF), thereby suppressing the expression of the auxin-induced gene [42,43]. Conversely, an excess auxin concentration triggers the binding of Aux/IAA with F-box protein-transport inhibitory response 1 (TIR1), which triggers degradation [44,45]. Furthermore, TIR1 and protein auxin-related F-box (AFB) are receptors for the nucleus associated with auxin-regulated genes. However, AFB1-3 has a higher affinity for 2,4-D than TIR1 [14].

The presence of 2,4-D likely promotes cell cycle processes by triggering the formation of cyclin-dependent kinase complexes and activating the binding of catalytic cyclin-dependent kinase-A (CDKA) and D-type cyclins (CYCD) [46]. The 2,4-D complex and CDKA/CYCD may be involved in initiating a phase transition in the cell cycle from growth-1 (G1) to synthesis (S) [47]. In low levels, auxin and cytokinin play a role in activating CDC25-like phosphatase, which is involved in the transition of the G2 cycle to mitosis [48]. Simultaneous activation of cell proliferation due to phytohormones then promotes increased biomass due to callus formation.

The increase in mass has an impact on the quantity and quality of callus metabolism. Furthermore, plant cells produce secondary metabolites of sugar compounds due to the derivation of the molecule glyceraldehyde-3 phosphate (G3P) [49]. During callus growth, more G3P is converted into sugars and then oxidized to produce adenosine triphosphate (ATP), an energy for cell division and callus addition. The composition and type of secondary metabolites usually differ depending on the type of tissue or organ and at a particular stage of development. This follows this study, where low concentrations of 2,4-D and kinetin (K2D2) in the callus *D. esculenta* produce the highest secondary metabolites, especially flavonoids. Plants have various types of sugars, including 3-deoxy-O-arabino-heptulosonate phosphate (DAHP), then undergo metabolism and are reduced to a shikimate form [50]. Shikimate also forms compounds from the phenolic group, aromatic amino acids (tryptophan, tyrosine, and phenylalanine) to primary metabolites.

The integration of kinetin and auxin plays a transcriptional activator to enhance/ the synthesis and activation of the phytochrome function, which is responsible for the response of plant cells to light response [41]. This can be seen in this study, where calluses cultured with lighting can produce biomass, and flavonoid compounds are higher than calluses in dark conditions. The presence of light may affect the activation of various proteins involved in the biosynthesis of secondary metabolites. Recent research has shown that the presence of light affects post-transcriptional regulation of gene expression of enzymes that make up the structure of flavonoids [51].

Constitutive photomorphogenic1 (COP1) protein acts as a negative nuclear regulator for the presence of light that binds to the suppressor of phyA (SPA) proteins to form COP1/SPA complexes localized in the cell nucleus [52]. In dark conditions, COP1/SPA will bind transcription factors such as elongated hypocotyl-5 HY5 and R2R3-MYBs for the ubiquitination process towards degradation through the 26S proteasome pathway. Meanwhile, in bright conditions, visible light activates photoreceptor proteins such as phytochrome (PHY) and cryptochromes (CRY) [53]. PHY and CRY proteins then form complexes with COP1/SPA and translocate those proteins out of the cell nucleus, allowing transcription factors. Flavonoid structural gene expression is directly regulated by the transcription factor R2R3-MYB which may be held by bZIP transcription factors such as HY5 [54].

Light triggers auxin degradation, but the presence of PGR exogen simultaneously substitutes phytohormone deficiency and plays a synergistic role in flavonoid biosynthesis. Nonetheless, it is essential to note that specific interactions and mechanisms on the synergistic effects between kinetin, 2,4-D, and light in flavonoid biosynthesis may vary depending on plant species and research context. Therefore, more research is needed to unravel the complex molecular interactions and signaling networks underlying these synergistic effects.

Conclusions

The kinetin and 2,4-D significantly increase the flavonoid content of *D. esculenta* callus culture under light conditions. In addition, the combination of 0.5 ppm kinetin and 0.5 ppm 2,4-D to *D. esculenta* callus cultures was observed to significantly increase growth and flavonoid content, especially kaempferol, isoflavone, quercetin, flavanone, and epigallocatechin. This result justifies the synergistic effect between kinetin and 2,4-D on the biosynthesis of secondary metabolite compounds. This study has the potential to open new avenues and perspectives on strategies for increasing the production of bioactive compounds using light in D. esculenta callus cultures. Further research needs to be developed to determine the quality of lighting based on light intensity and spectrum.

Acknowledgment

The authors thank the Directorate of Research and Community Service of the Ministry of Education and Culture of the Indonesian Republic for funding this research with contract number 9.14.4/UN37/PPK.4.4/2022.

References

[1] DS Retnowati, R Ratnawati and AC Kumoro. Nutritional characteristics and potential applications of flour prepared from Indonesian wild white yam (*Dioscorea esculenta* L.). *Reaktor* 2016; **19**, 43-8.

[2] JE Obidiegwu, JB Lyons and CA Chilaka. The *Dioscorea* genus (yam): An appraisal of nutritional and therapeutic potentials. *Foods* 2020; **9**, 1304.

[3] TH Gandhi, R Gokarn, SKN Kumar, AS Ravikrishna and BT Shridhar. QR code pharmacognostical and analytical standards of *Dioscorea Esculenta* Burkill. *J. Res. Tradit. Med.* 2017; **3**, 157-68.

[4] M Murugan and VR Mohan. *In vitro* antioxidant studies of *Dioscorea esculenta* (Lour). Burkill. *Asian Pac. J. Trop. Biomed.* 2012; **2**, S1620-S1624.

[5] WDR Putri, EI Riyanto, A Heliana, A Wellang and E Zubaidah. Optimization of microwave-assisted extraction (mae) time and material to solvent ratio of gembili (*Dioscorea esculenta*) water-soluble polysaccharides (WSP). *Industria Jurnal Teknologi Manajemen Agroindustri* 2021; **10**, 78-98.

[6] M Anwar, EJ Birch, Y Ding and AED Bekhit. Water-soluble non-starch polysaccharides of root and tuber crops: Extraction, characteristics, properties, bioactivities, and applications. *Crit. Rev. Food Sci. Nutr.* 2022; **62**, 2309-41.

[7] S Indrawati, LO Ginting and J Safuan. Chemical composition of *Dioscorea alata* L. and *Dioscorea esculenta* (Lour.) Burk. cultivars from Wakatobi Islands, Indonesia. *Int. J. Sci. Tech. Res.* 2020; **9**, 939-45.

[8] R Nazir, V Kumar, S Gupta, P Dwivedi, DK Pandey and A Dey. Biotechnological strategies for the sustainable production of diosgenin from *Dioscorea* spp. *Appl. Microbiol. Biotechnol.* 2021; **105**, 569-85.

[9] NS Aminah, R Hidayah and M Tanjung. Confusarin and nudol, two phenathrene group compounds, from *Dioscorea esculenta* L. and their antioxidant acitivities. *J. Chem. Tech. Metall.* 2017; **52**, 1135-9.

[10] B Kaur, S Khatun and A Suttee. Current highlights on biochemical and pharmacological profile of *Dioscorea alata*: A review. *Plant Arch.* 2021; **21**, 552-9.

[11] S Senanayake, K Ranaweera, A Bamunuarachchi and A Gunaratne, Proximate analysis and phytochemical and mineral constituents in four cultivars of yams and tuber crops in Sri Lanka. *Trop. Agr. Res. Ext.* 2013; **15**, 32.

[12] M Silalahi. *Dioscorea esculenta* (Lour.) Burkill: Uses and bioactivity. *Int. J. Biol. Pharmaceut. Sci. Arch.* 2022; **3**, 20-5.

[13] NA Habibah, S Safitri, YR Pratiwi, N Wijawati, F Musafa, ADS Puspitasari and A Yuniastuti. Callus induction from tuber of lesser yam (*Dioscorea esculenta*) on MS media supplemented by 2,4-D and kinetin. *J. Phys. Conf.* 2021; **1918**, 052029.

[14] Y Song. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *J. Integr. Plant Biol.* 2014; **56**, 106-13.

[15] NA Habibah, YU Anggraito, N Nugrahaningsih, S Safitri, F Musafa and N Wijayati. LC-MS based secondary metabolites profile of *Elaeocarpus grandiflorus* J.E. Smith. cell suspension culture using picloram and 2,4-dichlorophenoxyacetic acid. *Trop. J. Nat. Prod. Res.* 2021; **5**, 528-33.

[16] DP Gaucan, S Bhuju, J Lamichhane, R Shakya and MR García-Gil. Establishment of regenerative callus, cell suspension system, and molecular characterization of *Taxus wallichiana* Zucc. for the *in vitro* production of Taxol. *J. Appl. Pharmaceut. Sci.* 2021; **11**, 22-34.

[17] MA Ahanger, RA Mir, MN Alyemeni and P Ahmad. Combined effects of brassinosteroid and kinetin mitigates salinity stress in tomato through the modulation of antioxidant and osmolyte metabolism. *Plant Physiol. Biochem.* 2020; **147**, 31-42.

[18] G Hao, X Du, F Zhao, R Shi and J Wang. Role of nitric oxide in UV-B-induced activation of PAL and stimulation of flavonoid biosynthesis in *Ginkgo biloba* callus. *Plant Cell Tissue Organ Cult.* 2009; **97**, 175-85.

[19] C Nelimor, HY Sintim, AW Kena and R Akromah. Using surface response models to evaluate the effects of kinetin on *Dioscorea alata* propagated *in vitro*. *J. Agr. Sci. Tech.* 2017; **7**, 69-78.

[20] NA Habibah, S Moeljopawiro, K Dewi and A Indrianto. Flavonoid production, growth and differentiation of *Stelechocarpus burahol* (Bl.) hook. f. and th. cell suspension culture. *Pakistan J. Biol. Sci.* 2017; **20**, 197-203.

[21] NA Habibah, T Widiatningrum, YU Anggraito, ES Rahayu, K Mukhtar, N Wijayanti and F Mustafa. Growth of *Elaeocarpus grandiflorus* callus cultures in ms medium with various concentrations of growth regulators. *J. Phys. Conf.* 2019; **1321**, 2-7.

[22] H Li, A Radunz, P He and GH Schmid. Influence of different light intensities on the content of diosgenin, lipids, carotenoids and fatty acids in leaves of *Dioscorea zingiberensis*. *J. Biosci.* 2002; **57**, 135-43.

[23] RDS Handayani, I Yunus, M Sayuti and E Irawan. *In-vitro* callus induction of Durian (*Durio zibethinus* Murr.) leaves using kinetin and 2,4-d (dichlorophenoxyacetic acid). *J. Trop. Horticulture* 2019; **2**, 59.

[24] NA Habibah, S Safitri, YR Pratiwi, N Wijawati, F Musafa, ADS Puspitasari and A Yuniastuti. Callus induction from tuber of lesser yam (*Dioscorea esculenta*) on MS media supplemented by 2,4-D and kinetin. *J. Phys. Conf.* 2021; **1918**, 052029.

[25] ES Isabelle and E Florent. Effect of various growth regulators on growth of yam (*Dioscorea trifida* L.) *in vitro* shoot tips. *Afr. J. Biotechnol.* 2014; **13**, 1645-9.

[26] MH Bhat, M Fayaz, A Kumar, AA Dar and AK Jain. Development of an efficient micropropagation system for *Dioscorea bulbifera* L. and phytochemical profile of regenerated plants. *J. Genet. Eng. Biotechnol.* 2022; **20**, 107.

[27] R Quian-Ulloa and C Stange. Carotenoid biosynthesis and plastid development in plants: The role of light. *Int. J. Mol. Sci.* 2021; **22**, 1184.

[28] S Kapoor, R Raghuvanshi, P Bhardwaj, H Sood, S Saxena and OP Chaurasia. Influence of light quality on growth, secondary metabolites production and antioxidant activity in callus culture of *Rhodiola imbricata* Edgew. *J. Photochem. Photobiol. B Biol.* 2018; **183**, 258-65.

[29] R Khurshid, MA Ullah, D Tungmunnithum, S Drouet, M Shah, A Zaeem, S Hameed, C Hano and BH Abbasi. Lights triggered differential accumulation of antioxidant and antidiabetic secondary metabolites in callus culture of *Eclipta alba* L. *PLos One* 2020; **15**, e0233963.

[30] M Thakur, S Bhattacharya, PK Khosla and S Puri. Improving production of plant secondary metabolites through biotic and abiotic elicitation. *J. Appl. Res. Med. Aromat. Plants* 2019; **12**, 1-12.

[31] M Nazir, MA Ullah, M Younas, A Siddiquah, M Shah, N Giglioli-Guivarc'h, C Hano and BH Abbas. Light-mediated biosynthesis of phenylpropanoid metabolites and antioxidant potential in callus cultures of purple basil (*Ocimum basilicum* L. var *pturpurascens*). *Plant Cell Tissue Organ Cult.* 2020; **142**, 107-20.

[32] CC Lai, H Pan, J Zhang, Q Wang, QX Que, R Pan, ZX Lai and GT Lai. Light quality modulates growth, triggers differential accumulation of phenolic compounds, and changes the total antioxidant capacity in the red callus of *Vitis davidii*. *J. Agr. Food Chem.* 2022; **70**, 13264-78.

[33] B Biswal, B Jena, AK Giri and L Acharya. Monochromatic light elicited biomass accumulation, antioxidant activity, and secondary metabolite production in callus culture of *Operculina turpethum* (L.). *Plant Cell Tissue Organ Cult.* 2022; **149**, 123-34.

[34] A Jogawat, B Yadav, C Chhaya, N Lakra, AK Singh and OP Narayan. Crosstalk between phytohormones and secondary metabolites in the drought stress tolerance of crop plants: A review. *Physiol. Plantarum* 2021; **172**, 1106-32.

[35] J Lang, J Pankowski, P Grabarz, B Pluciński and P Jedynaz. Comparing the effects of different exogenous hormone combinations on seed-derived callus induction in *Nicotiana tabacum*. *Acta Biochim. Pol.* 2020; **27**, 449-45.

[36] GA Romanov. Perception, transduction and crosstalk of auxin and cytokinin signals. *Int. J. Mol. Sci.* 2022; **23**, 13150.

[37] JR Avilez-Montalvo, AO Quintana-Escobar, HA Méndez-Hernández, V Aguilar-Hernández, L Brito-Argáez, RM Galaz-Ávalos, MA Uc-Chuc and VM Loyola-Vargas. Auxin-cytokinin cross talk in somatic embryogenesis of *Coffea canephora. Plants* 2022; **11**, 2013.

[38] I Antoniadi, O Novák, Z Gelová, A Johnson, O Plíhal, R Simerský, V Mik, T Vain, E Mateo-Bonmatí, M Karady, M Pernisová, L Plačková, K Opassathian, J Hejátko, S Robert, J Friml, K Doležal, K Ljung and C Turnbull. Cell-surface receptors enable perception of extracellular cytokinins. *Nat. Comm.* 2022; **11**, 4284.

[39] GA Romanov and T Schmülling. On the biological activity of cytokinin free bases and their ribosides. *Planta* 2022; **255**, 27.

[40] HT Hallmark and AM Rashotte. Review - Cytokinin response factors: Responding to more than cytokinin. *Plant Sci.* 2019; **289**. 110251.

[41] H Xu, P Chen and Y Tao. Understanding the shade tolerance responses through hints from phytochrome a-mediated negative feedback regulation in shade avoiding plants. *Front. Plant Sci.* 2021; **12**, 813092.

[42] T Yamauchi, A Tanaka, H Inahashi, NK Nishizawa, N Tsutsumi, Y Inukai and M Nakazono. Fine control of aerenchyma and lateral root development through AUX/IAA- And ARF-dependent auxin signaling. *Proc. Nat. Acad. Sci. Unit. States Am.* 2019; **116**, 20770-5.

[43] J Luo, JJ Zhou and JZ Zhang. Aux/IAA gene family in plants: Molecular structure, regulation, and function. *Int. J. Mol. Sci.* 2018; **19**, 1-17.

[44] M Fendrych, M Akhmanova, J Merrin, M Glanc, S Hagihara, K Takahashi, N Uchida, KU Torii and J Friml. Rapid and reversible root growth inhibition by TIR1 auxin signalling. *Nat. Plants* 2018; **4**, 453-9.

[45] N Uchida, K Takahashi, R Iwasaki, R Yamada, M Yoshimura, TA Endo, S Kimura, H Zhang, M Nomoto, Y Tada, T Kinoshita, K Itami, S Hagihara and KU Torii. Chemical hijacking of auxin signaling with an engineered auxin-TIR1 pair. *Nat. Chem. Biol.* 2018; **14**, 299-305.

[46] JW Chandler. Auxin response factors. *Plant Cell. Environ.* 2016; **39**, 1014-28.

[47] P Vieira and JA Engler. Plant cyclin-dependent kinase inhibitors of the KRP family: Potent inhibitors of root-knot nematode feeding sites in plant roots. *Front. Plant Sci.* 2017; **8**, 01514.

[48] H Kobayashi, T Saito, K Sato, K Furusawa, T Hosokawa, K Tsutsumi, A Asada, S Kamada, T Ohshima and S Hisanaga, Phosphorylation of cyclin-dependent kinase 5 (Cdk5) at Tyr-15 is inhibited by Cdk5 activators and does not contribute to the activation of Cdk5. *J. Biol. Chem.* 2014; **289**, 19627-36.

[49] S Pachauri, S Chatterjee, V Kumar and PK Mukherjee. A dedicated glyceraldehyde-3-phosphate dehydrogenase is involved in the biosynthesis of volatile sesquiterpenes in Trichoderma virens—evidence for the role of a fungal GAPDH in secondary metabolism. *Curr. Genet.* 2019; **65**, 243-52.

[50] A Zabalza, L Orcaray, M Fernández-Escalada, A Zulet-González and M Royuela. The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots. *Pestic. Biochem. Physiol.* 2017; **141**, 96-102.

[51] M Qian, H Wu, C Yang, W Zhu, B Shi, B Zheng, S Wang, K Zhou and A Gao. RNA-Seq reveals the key pathways and genes involved in the light-regulated flavonoids biosynthesis in mango (*Mangifera indica* L.) peel. *Front. Plant Sci.* 2023; **13**, 1119384.

[52] DM Choi, SH Kim, YJ Han and JI Kim. Regulation of plant photoresponses by protein kinase activity of phytochrome A. *Int. J. Mol. Sci.* 2023; **24**, 2110.

[53] H Kang, TT Zhang, LL Fu, CX You, XF Wang and YJ Hao. The apple RING-H2 protein MdCIP8 regulates anthocyanin accumulation and hypocotyl elongation by interacting with MdCOP1. *Plant Sci.* 2020; **301**, 110665.

[54] E Cañibano, C Bourbousse, M García-León, BG Gómez, L Wolff, C García-Baudino, R Lozano-Durán, F Barneche, V Rubio and S Fonseca. DET1-mediated COP1 regulation avoids HY5 activity over second-site gene targets to tune plant photomorphogenesis. *Mol. Plant* 2021; **14**, 963-82.