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Vanilla Callogenesis with the Addition of Picloram and BAP under Dark and Light Conditions

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Abstract

Vanillin is a secondary metabolite in vanilla, with a 1%–3% content. Tissue culture is an effective alternative for producing plant secondary metabolites. This study aimed to analyze the effect of picloram and BAP under light and dark conditions on the callogenesis of vanilla leaf explants. A randomized block design was used with two factors: picloram concentration (0 ppm, 2.5 ppm, 5 ppm, 7.5 ppm) and BAP (0 ppm, 1 ppm, 2 ppm, 3 ppm), as well as lighting conditions (light and dark). Vanilla leaf explants were cultured on MS medium and incubated for three months. The observed parameters included the time for explants to curve, the percentage of curved explants, the time of callus formation, the percentage of callus-forming explants, and callus morphology. The results showed that PGRs type/concentration significantly affected the percentage of curved explants but had no significant effect on other parameters. Lighting and its interaction with PGRs had no significant effect. The best combination of picloram and BAP resulted in 100% curved explants but was less optimal for inducing callus formation in vanilla leaf explants. This study can open scientific insights into the combination of picloram and BAP and the effects of different lighting in vanilla leaf callogenesis.

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INTRODUCTION

Vanilla (*Vanilla planifolia* Andrews) is a cultivated plant with high economic value because it has a fragrant taste and aroma. Vanilla plants are one of Indonesia's agricultural export commodities used in the food, beverage, perfume, and pharmaceutical industries (Wahyudi *et al.,* 2021). Vanilla is rich in compounds, phenolics are the most important secondary metabolites produced by vanilla, which consist of vanillin (1%-3%), vanillic acid (0.1%-0.2%), p-hydroxy-benzaldehyde (0.1%-0.2%), and phydroxybenzoic acid (0.01%-0.02%) (Rojas-López & Cañizares-Macías, 2013). The vanilla plant is known to have antispasmodic, anti-inflammatory, and analgesic activities (Menon & Nayeem, 2013) .

Tissue culture is an effective alternative to produce secondary metabolite compounds of a plant through callus. Callus is a source of secondary metabolites that can be made in a shorter time. The nature of totipotency in plant cells can be maintained through tissue culture techniques, so that they can produce substances or compounds found in the mother plant (Fehér, 2019). Factors that affect biomass proliferation and accumulation of secondary metabolites in plant tissue culture are the type of explant, type of culture medium, type and level of carbohydrates, and the PGR levels (Murthy et al., 2014) .

Leaf explants used in tissue culture are based on the presence of meristematic cells capable of dedifferentiation, so they can be directed to induce callus (Lawrence et al., 2022). In addition, the role of plant growth regulators (PGRs) derived from a combination of auxins and cytokinins is very important in the induction of callus in vitro, including picloram and BAP. The effect of picloram as a growth regulator of auxinic herbicide class in callus formation is shown with a high percentage in some plants, namely in *Urochloa brizantha* as much as 69.2% at 2 ppm pikloram (Takamori et al., 2015), *Zingiber officinale* as much as 93.75% at 8 ppm picloram (Gnasekaran et al., 2023), and in *Allium ascalonicum* as much as 100% at 3 ppm pikloram (Ramadhan & Habibah, 2023). BAP, one type of cytokinin, has an important role in cell development at certain concentrations. Basal leaf explants *Vanda tricolor* Lindl. var. Pallida produces callus on half MS media with the addition of NAA 0.05 mg/L + BAP 0.01 mg/L (Hardjo et al., 2021). The addition of 1 mg/L BAP and 2 mg/L NAA was able to induce callus of *V. planifolia* on node segments (Sharma & Bora, 2015) .

Light intensity of culture in vitro has a great influence on the induction and growth of plant regeneration (Farhadi et al., 2017). Light can affect the optimization of PGRs as well as the adjustment of endogenous hormone levels (Deng et al., 2020). The importance of this research is that it focuses on finding the optimal formulation for the callogenesis of vanilla leaf explants in vitro. The purpose of this research is to analyze the effect of the administration of picloram and BAP in dark and light conditions on the callogenesis of vanilla leaf explants.

METHODS

This research was conducted at the Plant Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Semarang. This study used a randomized block design (RBD) consisting of two factors, namely the type/concentration of PGRs (picloram and BAP with 16 treatment combinations) and lighting conditions (dark and light).

Making Growing Media

Murashige and Skoog (MS) was weighed as much as 4.43 grams/liter; 30 grams/liter sugar; 0.1 grams/liter myo-inositol, and then put into an Erlenmeyer flask and added distilled water until the volume reached 1 liter. The pH of the media was set at 5,8. Plain agar of 8 grams/liter was added to the solution. The stock solution of picloram and BAP was added to the media according to the treatment (Table 1). The media was homogenized and cooked for 20 minutes. The media was poured into sterile culture bottles and sealed and sterilized using an autoclave at 121°C, pressure 1,5 atm for 20 minutes.

Table 1. Combination table

PGRs	Light	
	C_1	**C_2**
Z_1	Z_1C_1	Z_1C_2
Z_2	Z_2C_1	Z_2C_2
Z_3	Z_3C_1	Z_3C_2
Z_4	Z_4C_1	Z_4C_2
Z_5	Z_5C_1	Z_5C_2
Z_6	Z_6C_1	Z_6C_2
Z_7	Z_7C_1	Z_7C_2
Z_8	Z_8C_1	Z_8C_2
Z_9	Z_9C_1	Z_9C_2
Z_{10}	$Z_{10}C_1$	$Z_{10}C_2$
Z_{11}	$Z_{11}C_1$	$Z_{11}C_2$
Z_{12}	$Z_{12}C_1$	$Z_{12}C_2$
Z_{13}	$Z_{13}C_1$	$Z_{13}C_2$
Z_{14}	$Z_{14}C_1$	$Z_{14}C_2$
Z_{15}	$Z_{15}C_1$	$Z_{15}C_2$
Z_{16}	$Z_{16}C_1$	$Z_{16}C_2$

Description:
Z_1 = 0 ppm picloram + 0 ppm BAP
Z_2 = 0 ppm picloram + 1 ppm BAP
Z_3 = 0 ppm picloram + 2 ppm BAP
Z_4 = 0 ppm picloram + 3 ppm BAP
Z_5 = 2.5 ppm picloram + 0 ppm BAP
Z_6 = 2.5 ppm picloram + 1 ppm BAP
Z_7 = 2.5 ppm picloram + 2 ppm BAP
Z_8 = 2.5 ppm picloram + 3 ppm BAP
Z_9 = 5 ppm picloram + 0 ppm BAP
Z_{10} = 5 ppm picloram + 1 ppm BAP
Z_{11} = 5 ppm picloram + 2 ppm BAP
Z_{12} = 5 ppm picloram + 3 ppm BAP
Z_{13} = 7.5 ppm picloram + 0 ppm BAP
Z_{14} = 7.5 ppm picloram + 1 ppm BAP
Z_{15} = 7.5 ppm picloram + 2 ppm BAP
Z_{16} = 7.5 ppm picloram + 3 ppm BAP

C_1 = Light condition
C_2 = Dark conditions

Explant Subculture

Subcultures were conducted in an LAF with all equipment, media, and vanilla plantlets sterilized. Leaf explants were taken from the second to third leaf from the shoot tip and cut with an area of 1 cm x 1 cm. Explants were cultured in sterile bottles containing MS media containing PGRs according to the treatment.

Incubation

Culture bottles containing explants were randomly placed on the incubation rack. The light treatment was placed under a 1000 lux lamp lighting for 24 hours, and the dark treatment was placed on a dark shelf without lighting for 24 hours. The temperature in the incubation room ranged from 20-25°C, and the humidity was 52-58%.

Observation

The time of explant curving was observed when the explants started to show curving for the first time, by observing the day after culture (DAC). Explants were said to be curved when part of the explant was lifted or moved away from the surface of the media. The time of callus formation was calculated since the explants were cultured (DAC). The callus that appeared was calculated to be at least 1 mm in size. The percentage of curved and callus explants was observed at 3 months after culture. The percentage of curved and callus explants was calculated as follows:

$$\text{Percentage} = \frac{Number\ of\ curved\ or\ callus\ explants}{Number\ of\ explants\ cultured}\ x\ 100\%$$

Observations of callus morphology include the color and texture of the callus observed visually. Determination of callus color based on the Munsell Color Chart. While the texture of the callus formed, categorized based on callus crumb (friable) or compact callus (non-friable).

Data analysis

Quantitative data in this study will be previously tested for normality using the Kolmogorov-Smirnov test and homogeneity using the Levene test, with a significance value> 0.05. All data in quantitative data types do not meet the normal and homogeneous requirements, so a non-parametric test is carried out using the Kruskal-Wallis test. If the sig. value <0.05 means that there is a significant effect, so that further tests can be carried out using the Dunn test.

RESULTS AND DISCUSSION

The results showed that almost all treatments experienced curving in a time that was not much different. The results of data analysis using the Kruskal-Wallis test stated that all treatments were not significantly different from the time parameter of explant curving. The mean time for explants to curve in light conditions showed the lowest time of 14.00 DAC found in the control treatment, picloram 5 ppm + BAP 3 ppm, and picloram 7.5 ppm + BAP 1 ppm treatment. The highest mean time was 17.75 DAC in the treatment of picloram 0 ppm + BAP 2 ppm. The lowest mean value of time for explants to curve under dark conditions is 14.25 DAC is found in the treatment of picloram 2.5 ppm + BAP 0 ppm, picloram 2.5 ppm + BAP 3 ppm, and picloram 7.5 ppm + BAP 2 ppm. The highest mean time (17.50 DAC) was shown in the treatment of picloram 7.5 ppm + BAP 0 ppm (Table 1).

Table 1. Mean time for vanilla leaf explants to curve in each treatment concentration of picloram and BAP in light and dark conditions

PGRs Concentration	Mean time for explants to curve (DAC)	
	Light (day) *Mean ± SD*	**Dark (day)** *Mean ± SD*
Z_1	14.00± 0.00	14.50 ± 0.71
Z_2	14.25± 0.50	16.67 ± 3.78
Z_3	17.75 ± 3.78	16.33 ± 4.04
Z_4	17.00 ± 2.93	16.67 ± 3.78
Z_5	14.25 ± 0.50	14.25 ± 0.50
Z_6	14.25 ± 0.50	16.00 ± 3.36
Z_7	14.25 ± 0.50	14.50 ± 0.58
Z_8	14.25 ± 0.50	14.25 ± 0.50
Z_9	14.25 ± 0.50	14.33 ± 0.58
Z_{10}	14.25 ± 0.50	14.33 ± 0.58
Z_{11}	14.25 ± 0.50	16.33 ± 4.04
Z_{12}	14.00 ± 0.00	16.00 ± 2.71
Z_{13}	15.75 ± 3.50	17.50 ± 4.04
Z_{14}	14.00 ± 0.00	16.25 ± 3.20
Z_{15}	14.50 ± 0.58	14.25 ± 0.50
Z_{16}	16.25 ± 3.20	16.00 ± 3.37
Mean of each treatment	14.82	15.52

The results of the Kruskal-Wallis test showed that only the PGRs treatment (combination of picloram and BAP), which had a significant effect on the percentage of curved explants. The results of Dunn's test showed that the mean percentage was not significantly different, namely 100%, which was found in almost all treatments of the combination of picloram and BAP. All treatments of the combination of picloram and BAP were significantly different from the control treatment (picloram 0 ppm + BAP 0 ppm). In comparison, the treatment of picloram 0 ppm + BAP 3 ppm was not significantly different from all treatments (Table 2).

Table 2. Dunn's test on the mean percentage of curved vanilla leaf explants in the combined treatment of picloram and BAP.

PGRs Concentration	**Mean percentage of curved explants (%)** *Mean ± SD*
Z_1	50 ± 0.58^{b}
Z_2	88 ± 0.35^{a}
Z_3	88 ± 0.35^{a}
Z_4	75 ± 0.46^{ab}
Z_5	100 ± 0.00^{a}
Z_6	100 ± 0.00^{a}
Z_7	100 ± 0.00^{a}
Z_8	100 ± 0.00^{a}
Z_9	88 ± 0.35^{a}
Z_{10}	88 ± 0.35^{a}
Z_{11}	88 ± 0.35^{a}
Z_{12}	100 ± 0.00^{a}
Z_{13}	100 ± 0.00^{a}
Z_{14}	100 ± 0.00^{a}
Z_{15}	100 ± 0.00^{a}
Z_{16}	100 ± 0.00^{a}
Mean of each treatment	91.41

Notes: -Same letter annotations indicate no significant difference.
-Different letters indicate significant differences.

The results of the study on the parameter of callus explants were only found in the treatment in dark conditions with a combination of picloram 7.5 ppm + BAP 2 ppm at 35 DAC and picloram 2.5 ppm + BAP 3 ppm at 30 DAC (Table 3). Most of the explants did not show callus emergence until the end of the incubation period (90 DAC). The percentage of callus from both treatments was 25% each, which means that only one explant was callus. The morphology of the two calluses in each treatment is creamy yellow in color and friable in texture (Figure 1).

Table 3. Mean time for vanilla leaf explants to develop callus in each treatment concentration of picloram and BAP in light and dark conditions

PGRs Concentration	**Mean time for explants to develop callus (day)** *Mean ± SD*	**Mean percentage of explants with callus (%)** *Mean ± SD*
Z_8C_2	30.00 ± 0.00	25 ± 0.50
$Z_{15}C_2$	35.00 ± 0.00	25 ± 0.50

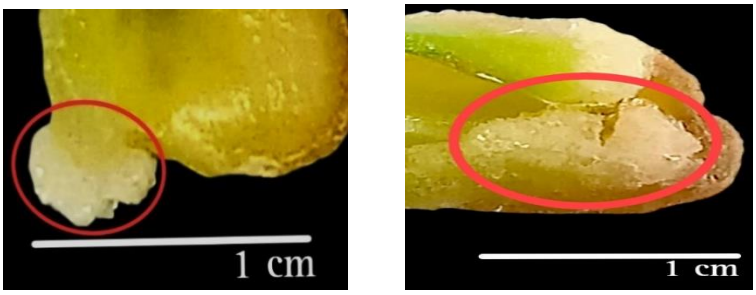

Figure 1. Creamy yellow and friable callus of vanilla formed from dark conditions. (a) Z_8C_2; (b) $Z_{15}C_2$.

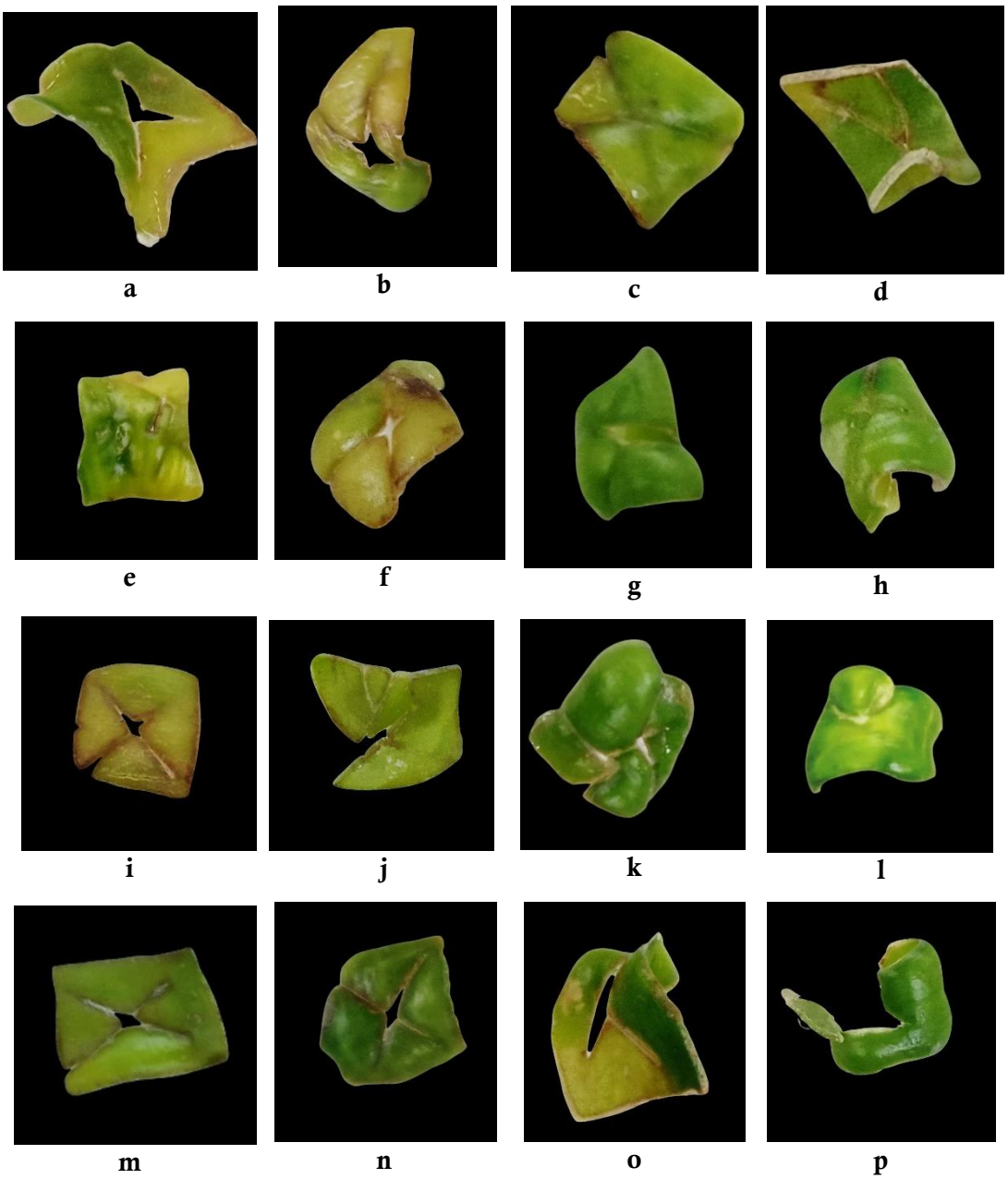

Figure 2. Curved explants under light conditions (90 DAC). (a) Z_1C_1; (b) Z_2C_1; (c) Z_3C_1; (d) Z_4C_1; (e) Z_5C_1; (f) Z_6C_1; (g) Z_7C_1; (h) Z_8C_1; (i) Z_9C_1; (j) $Z_{10}C_1$; (k) $Z_{11}C_1$; (l) $Z_{12}C_1$; (m) $Z_{13}C_1$; (n) $Z_{14}C_1$; (o) $Z_{15}C_1$; (p) $Z_{16}C_1$.

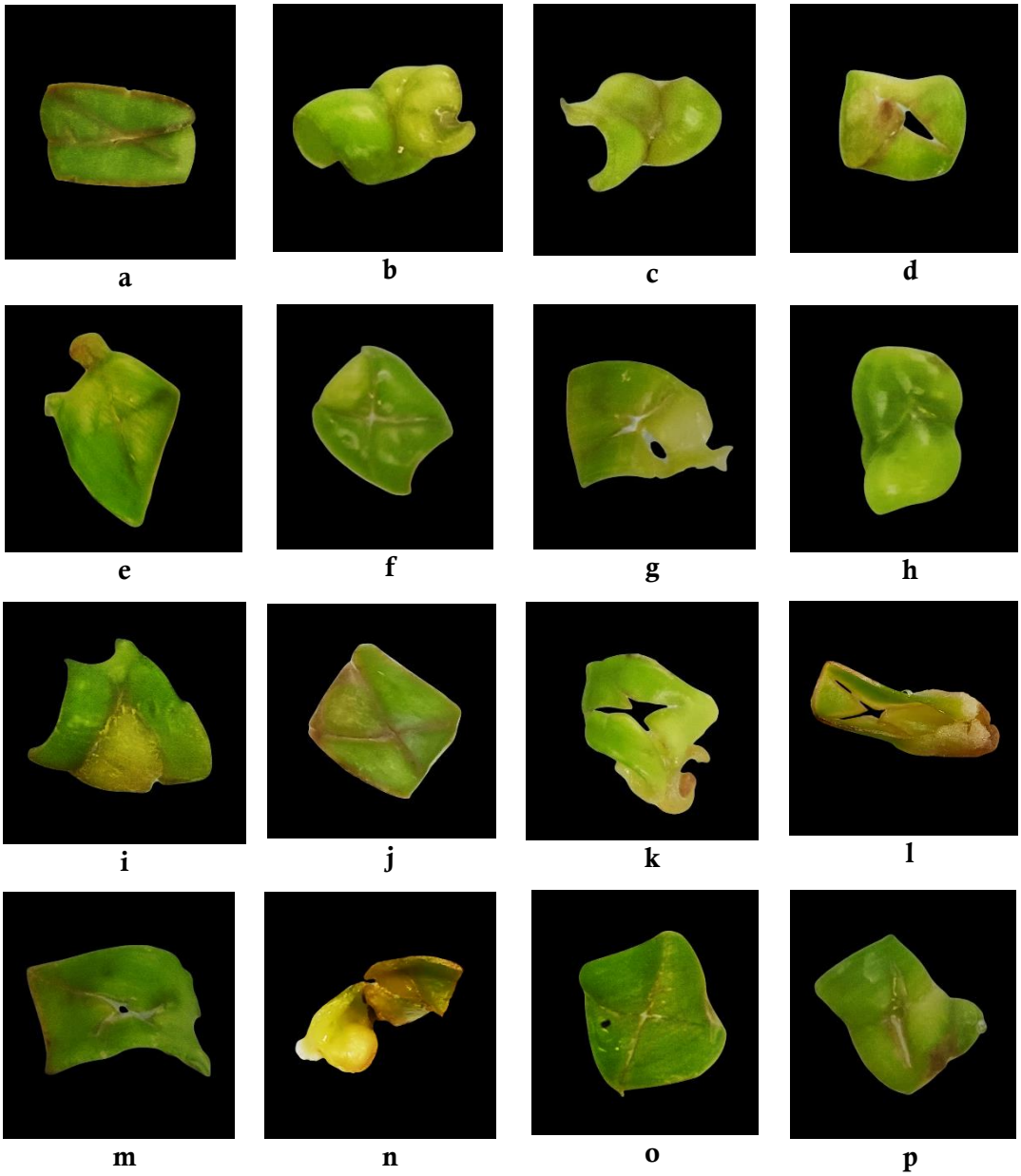

Figure 3. Curved explants under dark conditions (90 DAC). (a) Z_1C_2; (b) Z_2C_2; (c) Z_3C_2; (d) Z_4C_2; (e) Z_5C_2; (f) Z_6C_2; (g) Z_7C_2; (h) Z_8C_2; (i) Z_9C_2; (j) $Z_{10}C_2$; (k) $Z_{11}C_2$; (l) $Z_{12}C_2$; (m) $Z_{13}C_2$; (n) $Z_{14}C_2$; (o) $Z_{15}C_2$; (p) $Z_{16}C_2$.

The curved explants are a response to the media treated with PGRs and the influence of lighting conditions. Picloram, which is a synthetic auxin used in this study, plays a role in supporting cell enlargement in explants characterized by curving. Auxin increases the activity of the proton pump H^+-ATPase in the plasma membrane, which releases protons (H^+) into the wall matrix and causes apoplast acidification (pH 4,5-6). This mechanism, which causes the plasma membrane to hyperpolarize, is controlled by the protein small auxin up-RNA (SAUR) that can be induced by auxin (Du et al., 2020). Potassium ions are pumped into the cytosol when potassium channels are activated. This voltage is created due to the increased potassium concentration in the cytosol, which promotes water absorption and causes the cell membrane to expand (Majda & Robert, 2018). The effect of picloram in the curving process of explants was shown in a study conducted by Hassan et al. (2021) that the media with a

treatment combination of 2,0 mg/L picloram + 0,25 mg/L BA + 0,25 mg/L 2iP gave the highest results in the curving of spikelet explants *Phoenix dactylifera* L.

Cytokinin added together with auxin, gives a better effect on the percentage of explants curved. BAP is a synthetic cytokinin used in this study, it helps in cell division by increasing RNA and protein synthesis in various tissues (Anjani & Ratnawati, 2023). Cytokinin stimulates cell division and promotes cell expansion, mainly through the action of transcription factors such as Cytokinin-Responsive Growth Regulator (CKG), which promotes organ growth and regulates the cell cycle (Park et al., 2021). Cytokinin causes the expression of CKG, which controls the expression of several genes involved in macromolecular synthesis as well as genes involved in the cell cycle. Promoters of cell cycle-promoting factors such as WEE1, CDT1a, and DEL1 are physically bound by CKG. CKG is a mediator of the canonical cytokinin signaling cascade, especially downstream of B-type ARR2, which links to WEE1 in controlling cell size and cycle (Park et al., 2021). This is in line with the study conducted by Silva et al. (2017) showing that curving of pear leaf explants was observed in the early phase, then produced white callus from the edge of the wounded explants after three weeks of incubation in dark conditions with BA supplementation.

Light can influence hormonal and metabolic activities that support cell development. Plant cells' perception of light signals affects mRNA transcription, translation and stability, resulting in various physiological and metabolic reactions (Adil et al., 2019). Light and auxin promote cell enlargement by activating a central growth regulatory circuit involving ARF6, BZR1, and PIF4, which cooperatively regulate target genes. This interaction increases auxin sensitivity, facilitating cell elongation in response to light and hormonal signals (Oh et al., 2014). Before the stimulation of genes associated with cell cycle and cytoplasmic development, light causes the development of PIN1 polar membrane localization concomitant with auxin export (Mohammed et al., 2018)

Dark conditions were shown to favor higher auxin accumulation and cell hypertrophy. Enhanced cell hypertrophy, where cells undergo enlargement due to water accumulation and the formation of large vacuoles. Under dark conditions, phytochrome B (phyB) is inactivated, which allows phytochrome-interacting factor (PIF) to accumulate and regulate the transcription of its downstream targets. Among these are many auxin-related genes, resulting in increased auxin concentration in the cell through transcription of the YUCCA (YUC), changes in gene signaling auxin (AUX)/indole-3-acetic acid inducible (IAA), and increased auxin transport through proteins PIN-formed (PIN). These processes ultimately lead to cell elongation (Wit et al., 2016)

The results of the study on the parameters of callus explants and the percentage of callus explants, it is known that it comes from the treatment in dark conditions with the type/concentration of PGRs combined, namely picloram 7.5 ppm + BAP 2 ppm at 35 DAC and picloram 2.5 ppm + BAP 3 ppm at 30 DAC with a percentage of 25% each. The success of callus induction in tissue culture is strongly influenced by several factors, namely explant age, explant source, PGRs, culture conditions, and genetic variability among plant genotypes (Mohei et al., 2017). The amount of endogenous hormones in the explants, the type of exogenous hormones supplemented, the concentration of

cytokinin and auxin used, and their interaction with endogenous PGRs are some of the factors that determine the outcome of establishing an ideal hormonal gradient for the induction of callus

Picloram as an auxin herbicide can be used to stimulate callus (Phillips & Garda, 2019) . However, inappropriate concentrations or combinations of picloram and BAP may have caused an imbalance that failed to induce callus. The vanilla leaf explants used were also thought to be less responsive to either picloram or BAP. Because the response to auxins and cytokinins varies with each species or even plant tissue. Picloram is considered an auxinic herbicide that can induce endogenous auxin synthesis and has several stress effects (Pasternak & Steinmacher, 2024)

Research conducted by Ozel et al. (2022), showed that picloram with concentrations > 4.02 ppm caused more stress on the roots of *Allium cepa*. However, in contrast to research conducted by Gnasekaran et al. (2023), that picloram with a high concentration of 8 ppm is the optimal condition for callus induction of *Zingiber officinale* var. *rubrum*. Similarly, BAP can also inhibit callus formation in certain plant species if the concentration used is not appropriate. The findings of Khan et al. (2015) showed that the higher concentration of BAP (0.7 ppm) added inhibited callus formation in *Vitis vinifera* L., resulting in the lowest callus percentage of 31%. Research conducted by Chai et al. (2024) on vanilla root explants, BAP concentrations that are too high at > 2 ppm can reduce callus formation.

Morphological observations on the callus of vanilla leaf explants all produced callus with a crumbly texture and creamy yellow color (Figure 1). Callus color indicates the level of cell division activity, where white, cream, or whitish beige indicates that cell division is still actively occurring (Suhartanto et al., 2022). The treatment of auxin and cytokinin types/concentrations can cause diverse callus characteristics, including color and texture. Light intensity and duration can also affect callus color, especially in pigment production. Bright light tends to increase green color, while lower light intensity can make callus paler or whitish. The pale color is affected by the absence of light that is essential for photosynthesis and pigment production. Dark conditions affect metabolic processes in the callus, leading to reduced synthesis of compounds (Siddique & Islam, 2018)

Callus texture also indicates the quality of the callus. Callus texture can vary from friable to non-friable depending on the type of explant, base medium, PGRs and biotic and abiotic supplements in culture. Friable callus is formed through cell growth at small sites and loss of cell interactions influenced by the presence of auxin. Auxin stimulates cell elongation by increasing the plasticity of the cell wall to become loose, allowing water to go easily to the inner cell through osmosis, so that the cell extends. Thus, crumb callus contains a lot of water because the cell wall has not yet reached lignification, and the cells can be easily separated from each other (Wahyuni et al., 2020).

This research focuses on the interaction of the use of picloram and BAP in light and dark conditions, which have not been studied much before, especially for vanilla leaf callus induction. The results of this study are expected to open scientific insights into the combination of picloram and BAP and the effects of different lighting in inducing vanilla leaf callus.

CONCLUSION

The treatment of type/concentration of the PGRs picloram and BAP had a significant effect on the parameter of the percentage of curved explants, but had no significant effect on other parameters. Lighting and its interaction with the type/concentration of PGRs did not have a significant effect on all parameters. The combination of PGRs picloram and BAP at various concentrations is the optimal condition for the percentage of curved explants, which is 100%. The treatment of type/concentration of PGRs in both lighting conditions was not optimal for inducing callus in vanilla leaf explants. For further research, it is recommended to incubate in dark conditions, and further exploration is needed regarding the concentration of picloram and BAP to produce optimal vanilla leaf callus.

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