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Response of Rice Somatic Embryogenesis to Exogenous Melatonin About Its Role in Scavenging Reactive Oxygen Species

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ABSTRACT

The success rate of explant morphogenesis in plant breeding using tissue culture techniques is frequently plagued by browning due to the oxidation of phenolic compounds. The cumulated amount of reactive oxygen species (ROS) drives the oxidation of phenolic compounds. Melatonin is reported to take a part in modulating the regulation of antioxidant gene expression, reducing the accumulation of reactive oxygen species, and enhancing the efficacy of tissue culture. This study aims to determine the optimal melatonin concentration on the efficiency of plantlet regeneration and expression of the antioxidant resistance gene in rice callus. This study utilizes rice TN1, Gogo Niti II, Ketan Hitam, and Cigeulis cultivars. Melatonin at 0, 10, and 15 µM concentrations is supplemented in plantlet regeneration media. Rice antioxidant-related genes, Mn-SOD, Cu/ZnSOD, Cytosolic APX, CAT, GPOD, OsAPX, and OsCATA, expressed after melatonin supplementation. Melatonin concentration at 10 µM generates the highest expression of all tested genes in TN1 compared to other varieties. The cumulated amount of hydrogen peroxide (H_2O_2) shows that Melatonin has the potential to increase the proportion of plant regeneration in Cigeulis (90.48%) and Ketan Hitam (91.67%) varieties with a concentration of 10 µM and in TN1 (94.44%) and Gogo Niti II (80%) at a concentration of 15 µM.

INTRODUCTION

Plant tissue culture is a widely used technique in plant breeding of rice (*Oryza sativa* L.) that aims to rapidly increase selection efficiency and develop homozygous lines (Silva, 2010). The application of tissue culture as a technique for propagation is to generate shoot and root multiplication in numerous rice varieties (Purnamaningsih, 2006). In addition, tissue culture produces transgenic plants using the bacterium *Agrobacterium tumefaciens* to transform rice plants (Hiei et al., 1994). Producing transgenic plants in rice must have an efficient regeneration protocol as a prerequisite. However, as a key factor, some varieties usually have a low regeneration rate that needs improvement (Puhan et al., 2018). Moreover, rice tissue culture techniques are also used to study plant regeneration and the development of plant morphogenesis as models (Valdez et al., 1996).

One of the problems in tissue culture is oxidative stress, which occurs when the ratio of Reactive Oxygen Species (ROS) to antioxidants in plants is unbalanced (Yahraus et al., 1995). The existence of ROS can occur due to the continuous cleavage effect of the growth hormone used. As a result, plant cell proliferation is inhibited during morphogenesis if the content is too much or

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unbalanced. Besides inhibiting cell proliferation, the explant also changes color to brown, called browning. The browning of explants is caused by the oxidation of phenolic compounds, which results in tissue mortality and, thus, death in plant tissue (Onuoha et al*.,* 2011). These phenolic compounds can initiate ethylene production by plant tissues. Hence, the accumulation of large quantities of ethylene in vitro causes rapid differentiation and influences the growth and development of these explants (Biddington, 1992).

Using plant growth regulators or other compounds can improve the inhibition of morphogenesis in the plant tissue culture. Melatonin is frequently used in the tissue culture to modulate plant morphogenesis. Melatonin (N-acetyl-5-methoxytryptamine) is a member of the indoleamine group, stimulating various physicochemical responses in plants. Melatonin is a hormone that can regulate plant growth, aerial organ development, root morphology, and floral transition (Sun et al*.,* 2021). Melatonin even increases both abiotic and biotic stress resistance, such as when synthesized in plants to respond to abiotic stressors such as extreme temperature fluctuations, toxicity, soil salinity, drought, and biotic stressors such as fungal infections (Reiter et al*.,* 2015). Melatonin's other function is to overcome abiotic stress tolerance, which includes tolerance to heavy metals, ultraviolet radiation, and chemicals (Nawaz et al*.,* 2016).

Melatonin compounds have been extensively utilized in tissue culture, and multiple previous research studies have already demonstrated that Melatonin has the potential to act as a modulator of plant morphogenesis (Hardeland, 2015). Melatonin correlates with increased root formation, shoot formation in explants, and modulation of plant morphogenesis in vitro (Murch et al*.,* 2001). Melatonin can impact the auxin indoleacetic acid (IAA). This can happen because of the multifunctional signal molecule melatonin has to support plant vegetative growth and IAA auxin activity, impacting the growth rate of root and shoot organs (Sharif et al., 2018). Melatonin can also control tissue aging by nurturing plant tissue, regulating the formation of shoots and roots, and regulating the development of plant reproductive organs (Erland & Saxena, 2018). The function of Melatonin is also not limited to being a modulator of plant morphogenesis, photosynthetic efficiency, and metabolic rate but also as a free radical scavenger that is also known as an antioxidant, increasing plant tolerance by enhancing the activity of anti-antioxidant enzymes, lipid peroxidase, and regulating gene expression, which can influence plant performance (Fan et al., 2018).

The highest endogenous Melatonin can be found in chloroplasts and mitochondrial organelles, which also produce free radicals or ROS (Reactive Oxygen Species). Therefore, Melatonin in cells is also used to protect essential cellular organelles from oxidative stress and maintain their physiological functions (Tan et al., 2013). Melatonin also serves as an antioxidant, preventing and repairing cell damage in plants caused by free radicals, such as delaying aging during plant tissue differentiation. Exogenously applied Melatonin can activate enzyme activities such as catalase (CAT) and superoxide dismutase (SOD) (Zhang et al*.,* 2016). Melatonin activates the ascorbate peroxidase (APX) enzyme, which protects plants from oxidative stress (Bai et al*.,* 2020). Previous research already confirmed that melatonin treatment can increase the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in kiwi leaves, thereby delaying senescence, preventing chlorophyll degradation, and decreasing H_2 (Liang et al*.,* 2018). Each plant has a different level of cell regeneration. The treatment of the exogenous addition of Melatonin must be investigated further, particularly the preliminary studies that indicate a positive response to the explant morphogenesis process (Hardeland, 2015).

Morphogenesis, conversely, is heavily influenced by genetic background and physiological responses such as phenolic accumulation, ROS accumulation, and browning (discoloration), which induces ethylene and can inhibit the growth and development of explants. Melatonin, which activates key antioxidants and modulates and regulates antioxidant enzyme genes, is believed to be directly related to genes that can prevent ROS accumulation that induces browning. Studies indicate that Melatonin accelerates regeneration (Arnao & Hernández-Ruiz, 2018). Due to the part of Melatonin in antioxidants and antioxidant enzyme genes, the regeneration factor can be accelerated; however, it is possible that inhibition due to phenolic compounds, especially ethylene, is based on this, causing explants to carry out the regeneration process rapidly; however, this hypothesis requires further confirmation and study. Research advancements on melatonin compounds

in plants have opened up new research avenues, particularly at the cellular level, using in vitro techniques that allow researchers to study cellular-level morphogenesis by examining cell proliferation and differentiation during organogenesis and the capacity of cells to regenerate. This study was conducted to determine the optimal concentration of Melatonin on the success of plantlet regeneration and the expression level of the antioxidant resistance gene in rice callus.

MATERIALS AND METHODS

This study was established from January-June 2022 in the Agrotechnology Laboratory, Faculty of Agriculture, Universitas Jember, East Java, Indonesia. The rice seeds were obtained from Balai Besar Penelitian Tanaman Padi Subang (BB Padi).

Explant Preparation and Callus Induction

Rice seeds (TN1, Gogo Niti II, Ketan Hitam, and Cigeulis) were sterilized with a 1% sodium chloride solution shaken at 120 rpm for 30 minutes using an orbital shaker. The rice seed was then rinsed three times into sterile water and desiccated. The seed was planted in an induction medium. To make an induction medium mix the 4.14 x 10^{-3} kg/l of MS medium, 2 ppm of 2,4-D, 3 x 10^{-2} kg of sucrose, and 4 x 10^{-3} kg of gelrite (Safitri et al., 2016). The induction medium was pH 5.8 and autoclaved at 121°C and 15 psi for 30 minutes. Under the Laminar Air Flow (LAF) Cabinet, between 20 and 25 ml of the medium were deposited into a petri dish and then used to plant seed explants. The culture will go into dark incubation at 25°C for 4 weeks. The percentage of performed callus (Shahsavari et al., 2010) and the first day the callus appeared can be observed with a microscope. The observations included the embryogenic callus's color, structure, and shape, which performs somatic embryogenesis. The callus size was measured with ImageJR. The percentage of performed callus was measured as formula 1:

$$\text{Percentage of performed callus} = \frac{\text{Total number of explants forming calli}}{\text{Number of explants}} \times 100\%. 1)$$

H_2O_2 Content

The 10^{-4} kg of callus were homogenized using 10^{-4} l of 0.1% TCA solution. The homogeneous callus sample was transferred into a 1.5 x 10^{-3} l microtube and centrifuged at 12,000 rpm for 15 minutes to produce the supernatant. Then 5 x 10^{-7} l was taken, 4 x 10^{-7} l 10 mM phosphate buffer pH of 7, and 10^{-3} l of 1M Potassium Iodide were added. After that, the solution was incubated for 30 minutes at room temperature and measured with a spectrophotometer at 390 nm (Christou et al., 2014).

Plant Regeneration

An embryogenic callus 8 weeks old was then subcultured into tubes of regeneration media for shoot elongation. According to Safitri et al. (2016), regeneration media contains 4.41 x 10^{-3} kg/l MS medium, 1 ppm NAA, 2 ppm Kinetin, 2 x 10^{-3} kg/l Casein Hydrolyzate, sucrose 3 x 10^{-2} kg, gelrite 4 x 10^{-3} kg, and then mixed with each melatonin concentration (0, 10, 15 µM) as treatment. Each treatment was replicated three times, with each replicate consisting of a calli. The calli was then incubated with 16/8 (light/dark) light irradiation at 27°C. The response was collected based on the performance of greenspot formation (formula 2), percentage of plant regeneration (formula 3), and callus phase performance, such as globular, scutellar, and coleoptile phases, in the second and fourth weeks after treatment. The total number of growing explants was counted in the second week after treatment. The percentage of plant regeneration was established based on (Karthikeyan et al., 2009).

$$\text{Percentage of Green spot} = \frac{\text{Number of green spot on callus}}{\text{Total number of explant}} \times 100\% \ldots\ldots 2)$$

$$\text{Percentage of Plant regeneration} = \frac{\text{Number of regenerated calli}}{\text{Number of induced calli}} \times 100\% . 3)$$

Gene Expression Analysis

The genes OsAPX1, OsCATA, Mn-SOD, Cu/ZnSOD, CAT, GPOD, and Cytosolic APX exhibited expression (Table 1). Callus was collected two weeks and four weeks after culturing on a regeneration medium that consisted of melatonin treatment. Then, it was used as a sample and processed for gene expression analysis, consisting of RNA isolation, cDNA synthesis, and PCR. Total RNA was extracted from the callus using the Ribospin™ Plant Kit method (GeneAll), and cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix method (Toyobo). The PCR amplification profile consisted of an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The amplified PCR product was electrophoresed on a 2% agarose gel

stained with Greenstar and then visualized with a UV transilluminator to get a visual presentation.

Data Analysis

Research data were analyzed using ANOVA. If there was a significant difference between the treatments, then the DMRT at the 5% significance level was used as a further test. The data obtained from the visualization of gel electrophoresis results was analyzed using a quantitative descriptive analysis to describe expression level with a visual presentation.

RESULTS AND DISCUSSION

Callus Induction

The percentage of performed callus and callus size of several varieties on MS media with adding 2 ppm of 2,4-D hormone (Table 2) showed different results. The rice var. Gogo Niti II showed the highest percentage of explant capable of induction and callus size compared to Ketan Hitam but was not significantly different from Cigeulis and TN1. The callus induction rate for the Gogo Niti II and Cigeulis varieties reached 93.18% and 89.11 %, respectively, in Table 2. The difference in the percentage of callus induction and callus size of different varieties can be influenced by factors such as the background genetics of genotypes in roses (Nguyen et al*.,* 2020). This indicates that rice varieties have affected the induction level and the callus's size.

Callus Morphology

The callus morphology affected the callus's development in somatic embryogenesis. It is because callus performance can be utilized to predict a cell's ability to regenerate early on (Nabilah et al., 2022). Only the embryogenic calli type can become somatic embryogenic calli and be used to become plantlets (Ming et al*.,* 2018). Based on these conditions (Fig. 1), the callus of the var. TN1, var. Gogo Niti II, and Cigeulis are friable with white callus shiny bones and are categorized as somatic embryogenesis.

Table 1. Sequence primers for antioxidant gene expression analysis

Genes	Primer	Source
Mn-SOD	Forward: 5' GGA AAC AAC TGC TAA CCA GGA C 3' Reverse: 5' GCA ATG TAC ACA AGG TCC AGA A 3'	Kim et al., 2007
Cu/ZnSOD	Forward: 5' CAA TGC TGA AGG TGT AGC TGA G 3' Reverse: 5' GCG AAA TCC ATG TGA TAC AAG A 3'	Kim et al., 2007
Cytosolic APX	Forward: 5' AGT ACA TTG CCC GTG GTA CTC T 3' Reverse: 5' CGC ATT TCA TAC CAA CAC ATC T 3'	Kim et al., 2007
CAT	Forward: 5' CAT CTG GCT CTC CTA CTG GTC T 3' Reverse: 5' CAG GAG AAA CGT GTC TTC AGG T 3'	Kim et al., 2007
GPOD	Forward: 5' ACC GTG AGC GAG GAC TAC CT 3' Reverse: 5' AGC GTC AAG TGA GCC TTA GC 3'	Prakasha & Umesha, 2016
OsAPX1	Forward: 5' CCA AGG GTT CTG ACC ACC TA 3' Reverse: 5' CAA GGT CCC TCA AAA CCA GA 3'	Mun et al., 2017
OsCATA	Forward: 5' CGG ATA GAC AGG AGA GGT TCA 3' Reverse: 5' AAT CTT CAC CCC CAA CGA CT 3'	Mun et al., 2017
OsActin	Forward: 5' TCC ATC TTG GCA TCT CTC AG 3' Reverse: 5' GTA CCC GCA TCA GGC ATC TG 3'	Yang et al., 2008

Table 2. Percentage of callus induction (%) and callus size (mm)

Rice Varieties	Percentage of callus induction (%)	Callus Size (mm)
TN1	80.00 ± 15.14a	6.00 ± 0.60b
Gogo Niti II	93.18 ± 7.03a	7.16 ± 0.98a
Ketan Hitam	44.17 ± 10.87b	4.86 ± 0.41c
Cigeulis	89.11 ± 9.01a	5.53 ± 1.06bc

Remarks: The numbers followed by lowercase letters are the same in one column, meaning a significant difference in the DMRT mean difference test with a 95% confidence level

In contrast, the callus of the Ketan Hitam has a yellow color, a thicker texture, is dense, and is not as shiny as the other three varieties. The morphological characteristics of callicallus can also be classified into four main groups, such as I) white crumbly callus with shiny bones, II) yellow crumb callus, III) denser brownish yellow callus, and IV) denser brown (rhizogenic) callus (Visarada et al., 2002). These results are strongly influenced by genetic factors, media composition, and the interaction between the two factors. Previous studies have reported that the embryogenic callus characteristic has a yellowish-white color, and this condition is consistent with the callus in this study (Sahoo et al., 2011). Based on these conditions (Fig. 1), the callus of the varieties TN1, Gogo Niti II, and Cigeulis can be included in category I, namely, white callus crumbs with shiny bones. In contrast, the callus of the Ketan Hitam variety is included in category II with a yellow callus color and a thicker texture, dense and not too shiny when compared to the other three varieties.

Embryogenesis Somatic Development

The type of explant, parental genotypes, and physiological conditions can influence the development of somatic embryogenesis. The value of this success can be increased by applying exogenous hormones, one of which is Melatonin. Differences in application doses of exogenous Melatonin will impact the formation time of the somatic embryogenesis developmental phase from globular to coleoptile phase. The concentration can increase the day formation of callus, while the lowest concentration can inhibit the somatic embryogenesis process, according to the discussion on the differences in doses. Exogenous application of Melatonin at a concentration of 10 µM accelerated the formation of the globular phase in all rice varieties tested. However, this does not happen in TN1, equivalent to the concentration of 15 µM. The concentration of 15 µM melatonin also can accelerate the formation of the globular phase (Fig. 2), and the slowest growth was a concentration of 15 µM. This is consistent with the findings of Zhao et al. (2015), who discovered that higher melatonin concentrations inhibit growth, whereas doses as low as ≤ 10 µM promote yields.

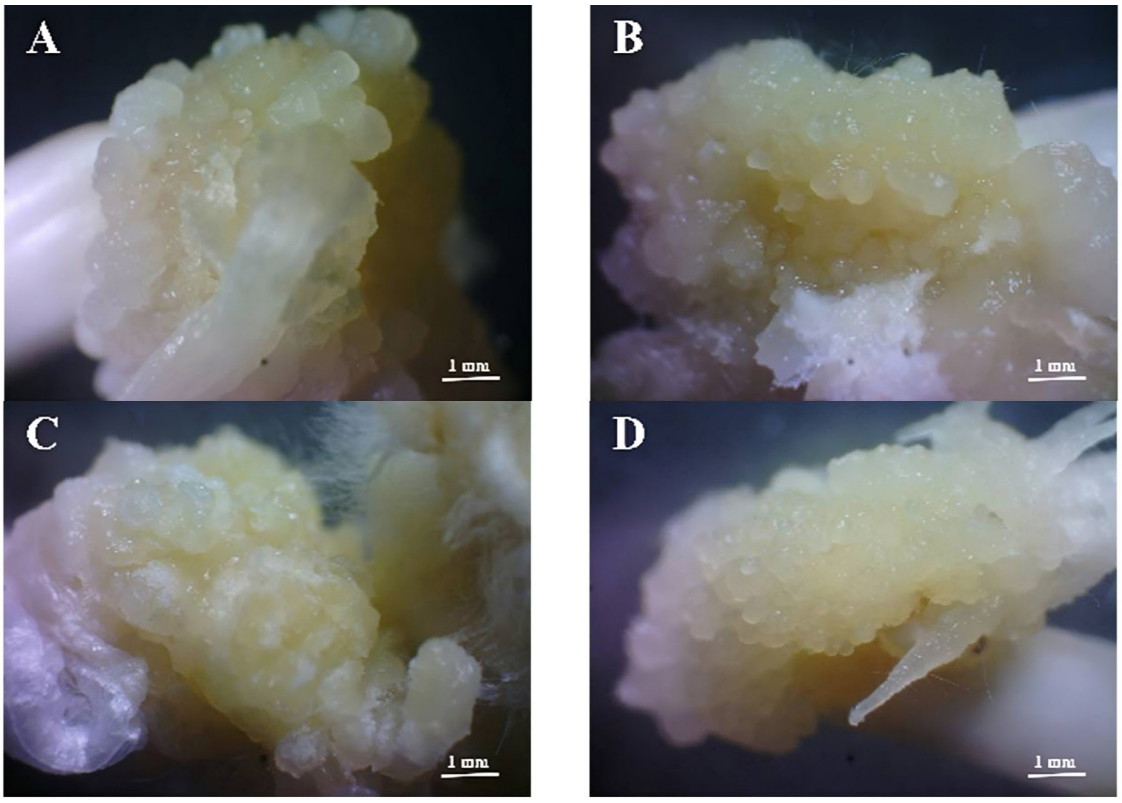


Fig. 1. Callus morphology of four rice varieties (A) TN1, (B) Gogo Niti II, (C) Ketan Hitam, and (D) Cigeulis after 4 weeks on callus induction medium (scale bars = 1 mm)

This statement is also supported by Duran et al. (2019), who applied 0, 100, and 200 µM to a culture of sweet basil. At 0 µM, culture regeneration could grow well; at 100 µM, it could still grow; however, growth was inhibited at 200 µM. In the scutellar phase, it was found that differences in exogenous melatonin concentration had a significant effect on the scutellar formation time (days). The concentration of 10 µM in TN1, Gogo Niti II, and Cigeulis varieties formed more rapidly in the scutellar phase than in other concentrations. However, this is not the case for the Ketan hitam rice variety, which has a faster growth rate at a concentration of 0 µM than 10 µM and 15 µM (Fig. 3). Various concentrations of Melatonin are applied to the regeneration medium to generate the coleoptilar phase. Supplementing with 10 µM melatonin in a regeneration medium significantly accelerates the colleoptilar phase in all varieties. (Fig. 4).

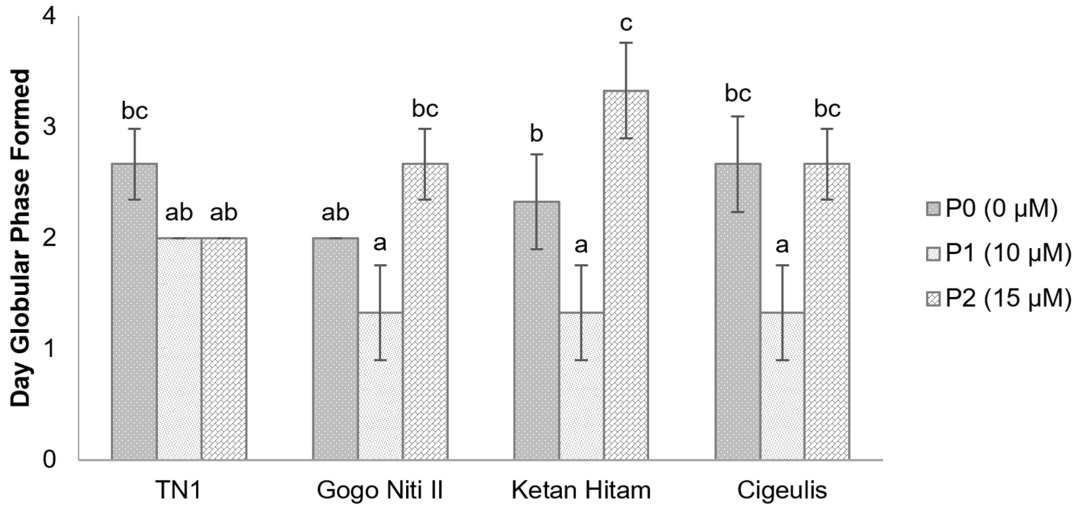


Fig. 2. The difference in the day of the formation of the globular phase due to differences in melatonin concentrations. The difference in letters shows a significant effect through the DMRT test ($p \leq 0.05$; $n = 5$)

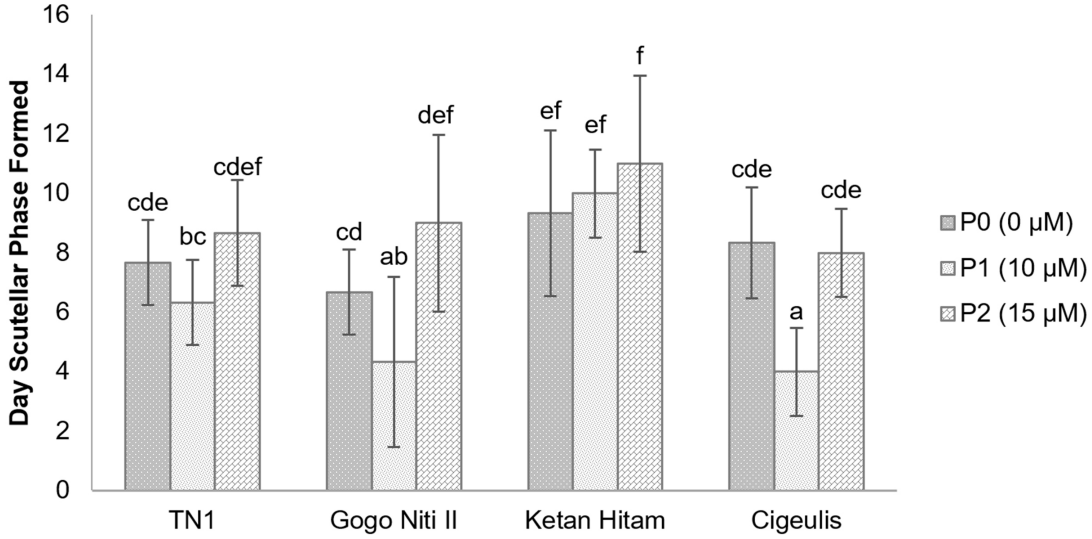


Fig. 3. The difference in the day of the formation of the scutellar phase due to differences in melatonin concentrations. The difference in letters shows a significant effect through the DMRT test ($p \leq 0.05$; $n = 5$)

Green Spot Percentage (%) and Plant Regeneration (%)

Four-week-old rice callus that had been subcultured in regeneration media were treated with melatonin concentrations of 0 µM, 10 µM, and 15 µM, and incubated with 16/8 hours of light (light/dark) light for 6 weeks. Observations about the percentage of green spots happen in the second (Fig. 5) and fourth weeks (Fig. 6). Altough, the observations are still conducted into the sixth week (Fig. 7) for observation of plantlet purpose. In the second week, the culture still did not show green spot or phase changes; in the fourth week, it is seen with the Gogo Niti var., which experienced the acceleration of the phase to become the fastest coleoptilar, and the sixth week the culture already become planlet. Table 3 demonstrates that differences in exogenous melatonin concentrations significantly impact green spot formation and plant generation. The exogenous concentration of 10 µM melatonin induces the highest percentage of green spots on the TN1, Gogo Niti II, and Cigeulis varieties, whereas 15 µM melatonin has the greatest effect on the TN1 variety. All varieties with a concentration of 0 µM melatonin or without treatment have the lowest incidence of green spots. Based on Table 3, the percentage of plant regeneration shows that Melatonin affects the variable rate of plant regeneration and that each variety background has a specific optimal concentration-response.

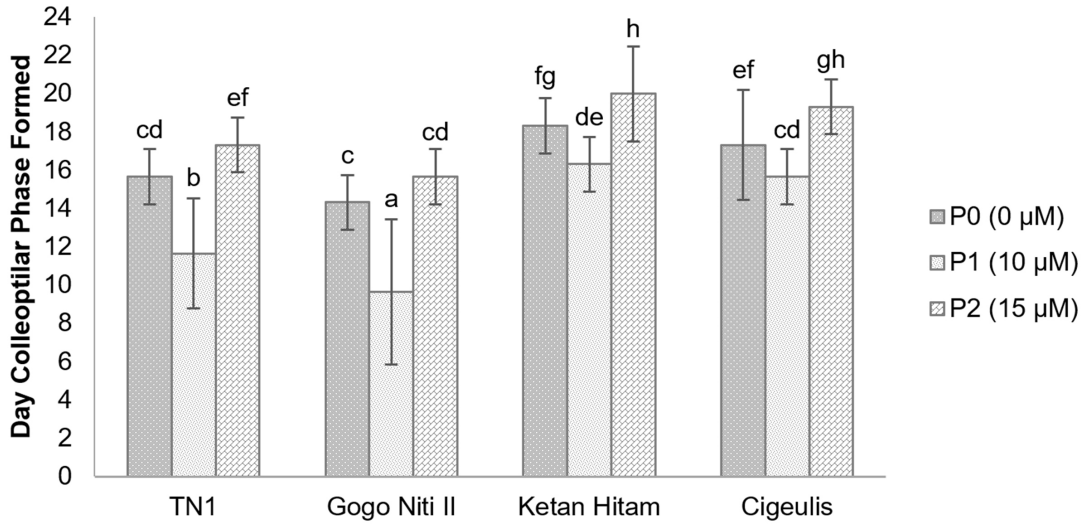


Fig. 4. The difference in the day of the formation of the colleoptilar phase due to differences in melatonin concentrations. The difference in letters shows a significant effect through the DMRT test ($p \leq 0.05$; $n = 5$)

Table 3. Percentage of green spot (%) and plant regeneration (%)

Varieties	Green Spot (%)			Regeneration (%)		
	P0 (0 µM)	P1 (10 µM)	P2 (15 µM)	P0 (0 µM)	P1 (10 µM)	P2 (15 µM)
TN1	31.75 ± 7.55 c	61.14 ± 4.93 a	58.25 ± 7.55 A	83.33 ± 14.43 a	85.71 ± 14.29 A	94.44 ± 9.62 A
Gogo Niti II	31.75 ± 7.55 c	62.29 ± 4.93 a	41.75 ± 13.97 b	91.67 ± 14.43 a	95.24 ± 8.25 A	80.00 ± 0.00 A
Ketan Hitam	33.51 ± 7.55 c	43.08 ± 10.92 b	37.55 ± 9.85 bc	53.33 ± 11.55 b	91.67 ± 14.43 a	58.33 ± 14.43 b
Cigeulis	33.51 ± 7.55 c	62.29 ± 4.93 a	41.75 ± 13.97 b	83.3 ± 14.43 a	90.48 ± 8.25 A	53.3 ± 11.55 B

Remarks: The numbers followed by lowercase letters are the same in one column, meaning a significant difference in the DMRT mean difference test with a 95% confidence level

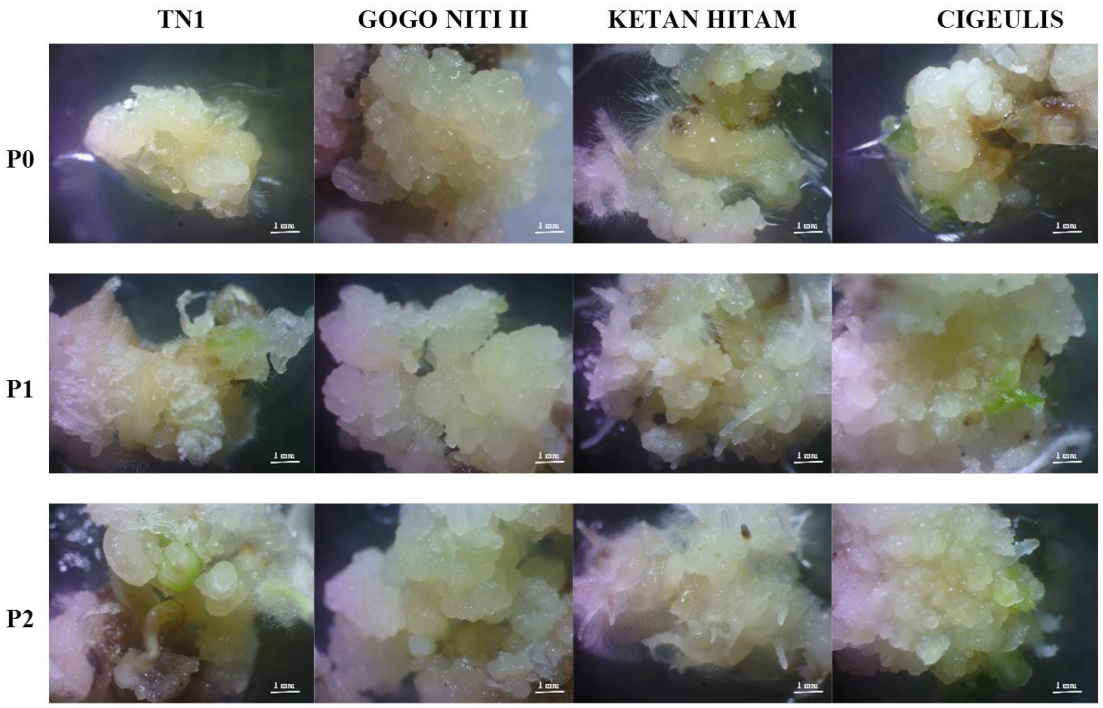


Fig. 5. Callus formation of four rice varieties in the second week after subculture to regeneration media with different melatonin concentrations. (P0) 0 µM Melatonin; (P1) 10 µM Melatonin; (P2) 15 µM Melatonin. (scale bars = 1 mm)

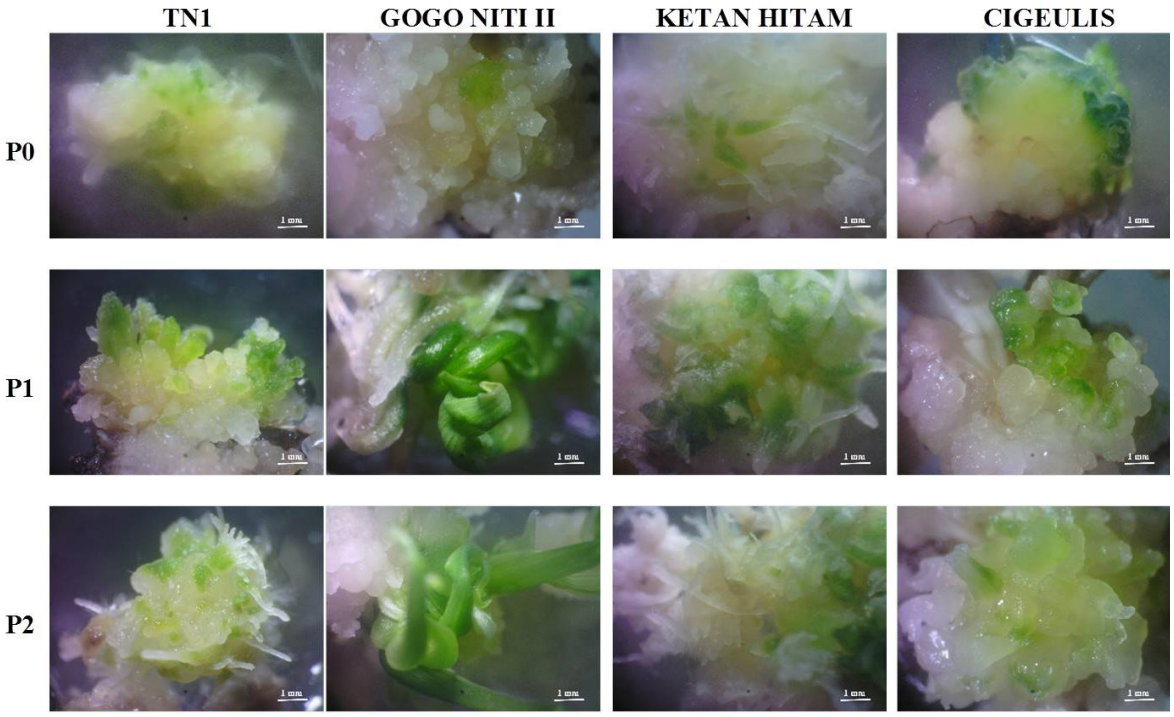


Fig. 6. Callus formation of four varieties of rice plants at (fourth week after subculture to regeneration media with different concentrations of Melatonin. (P0) 0 µM Melatonin; (P1) 10 µM Melatonin; (P2) 15 µM Melatonin. (scale bars = 1 mm)

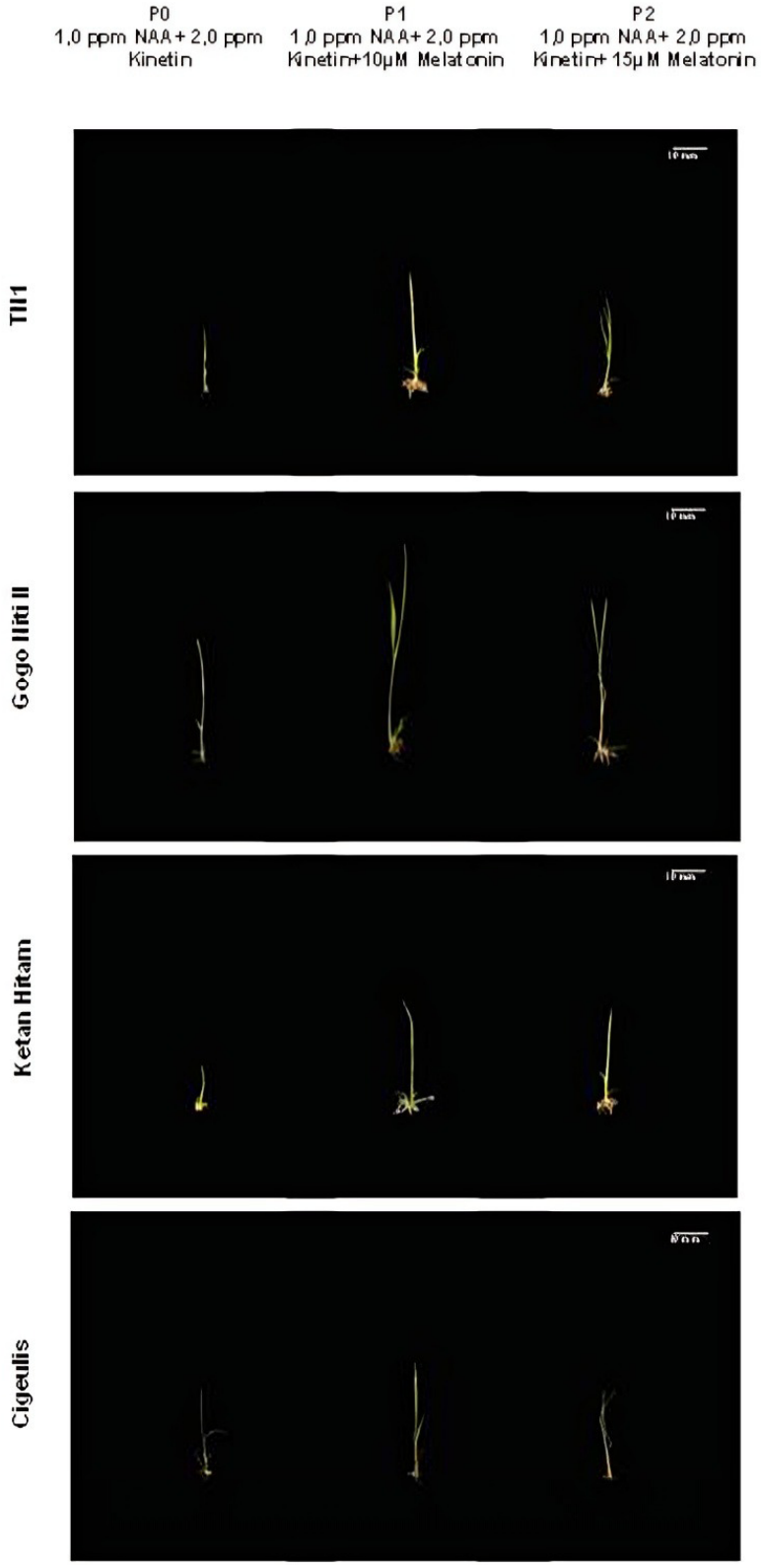


Fig. 7. Characteristics of regenerated plantlets after the sixth week in regeneration media with different Melatonin concentrations (scale bars = 10 mm)

Green spot is a variable that has a positive correlation with shoot regeneration. Besides, calluses with green spots will grow faster and produce more green spots than ordinary calli. Callus in the green spot phase in continuing the green spot and shoots requires growth regulators such as auxins (Nabors et al., 1982). Melatonin has a function similar to auxin, promoting cell development, organogenesis, and plant growth (Shi et al., 2016). Callus's success in forming a green spot on the regeneration media indicated photosynthesis occurred when placed under long irradiation. The formation of green spots based on Arnao & Hernández-Ruiz (2018) is one of the important phenomena to observe since it is an indicator of the plant regeneration phase. Parameters of plant regeneration can be determined by the number of plantlets that grow on the regenerated callus. Based on Table 3, the percentage of plant regeneration shows that Melatonin affects the variable rate of plant regeneration and that each variety background has a specific ideal concentration-response. As a result, Gogo Niti II var. shows the best results compared to other varieties in the 10 µM treatment for the percentage of callus induction, green spots, and plant regeneration. The same thing also happened in the study of Mostafiz et al. (2018), where the high percentage of callus induction also results in a high percentage of plant regeneration because each variety responds to different temperature treatments based on its genetic background.

Content of Hydrogen Peroxide (H_2O_2)

Fig. 8 shows that all rice varieties treated with exogenous Melatonin at different concentrations impact the H_2O_2 content during a 2-week-old callus culture in regeneration media. The concentration of Melatonin that can suppress the H_2O_2 content was 10 µM in all varieties compared to the control. The TN1 variety treated with the addition of Melatonin at a 10 µM concentration showed the lowest value. These values indicated that exogenous Melatonin at a concentration of 10 mM can suppress the H_2O_2 content well in rice calli on regeneration media. High H_2O_2 values are found at a concentration of 15 µM compared to the control, so this concentration was ineffective in suppressing oxidative stress in rice callus. Based on (Fig. 9) the treatment of melatonin concentrations experienced different H_2O_2 values during callus culture in all varieties aged 4 weeks in regeneration media. The fourth week shows the value of the H_2O_2 content compared to the second week. The H_2O_2 value in the fourth week shows the lowest value at 10 µM melatonin concentration in all varieties compared to the control. The Gogo Niti II variety at a melatonin concentration of 10 µM shows the lowest H_2O_2 value, suppressing oxidative stress, followed by the TN1, Gogo Niti II, and Cigeulis varieties. The Cigeulis variety, with a melatonin concentration of 15 µM has a lower value than the control, while the Gogo Niti II variety has the same value as the control.

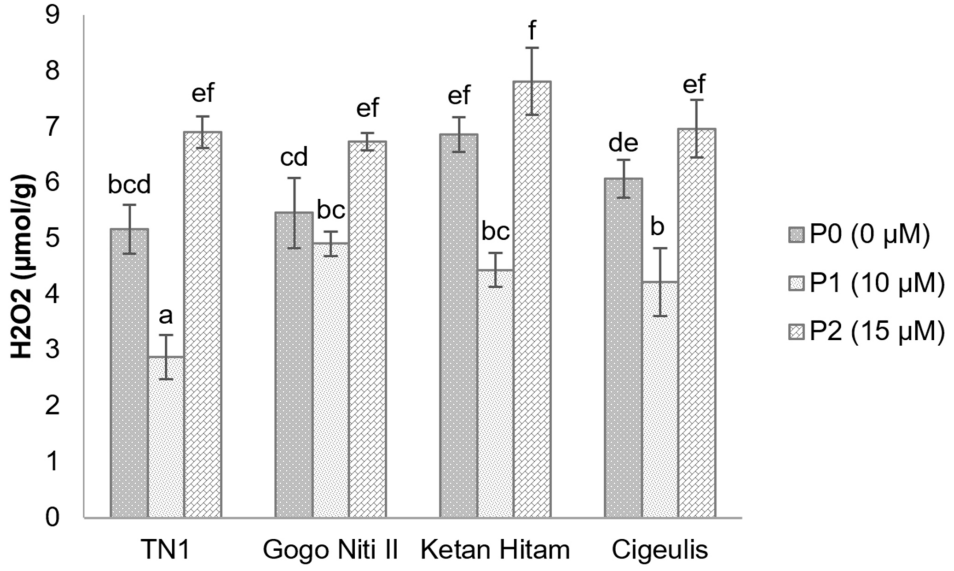


Fig. 8. Hydrogen peroxide content in all rice varieties treated with Melatonin in the second week. The difference in letters shows a significant effect through the DMRT test ($p \leq 0.05$; $n = 5$)

However, the TN1 and Ketan Hitam varieties at 15 µM have a high H_2O_2 value compared to the controls, so they can suppress oxidative stress that occurs in calli in the regeneration medium. Based on hydrogen peroxide content analysis, In 15 µM of melatonin concentration, the cigeulis variety had a lower result than the control, The Gogo niti II variety had the same result as the control, and then the TN1 and Ketan Hitam varieties had a higher content than the control. In all varieties, 10 µM of Melatonin could suppress the H_2O_2 content.

Antioxidant Gene Expression Analysis

Based on the antioxidant gene expression analysis (Fig. 10 and Fig. 11), the result shows that the level of gene expression in rice plants treated with Melatonin at three levels (0, 10, and 15 µM) increased, increasing all gene expression related to antioxidant enzymes in general. Gene expressions of MnSOD, Cu/ZnSOD, Cytosolic APX, CAT, GPOD, OsAPX, and OsCATA are used as research parameters to determine the response of antioxidant enzyme activity. Meanwhile, the OsActin gene served as a housekeeping gene that was used as an internal controller for the analysis of gene expression studies (Ubaidillah, et al., 2023).

Based on the results of the data (Fig. 10), the antioxidant gene expression level of the second week shows that the MnSOD gene in all rice varieties with P1 (10 µM) treatment is expressed higher than P0(0) and P2(15 µM). Meanwhile, the MnSOD gene in the fourth week (Fig. 11) in P2 treatment is expressed higher than P0 and P1. The Cu/ZnSOD gene in all rice varieties treated with P1 is expressed more strongly than in P0 and P2, whereas, in the fourth week, the gene expression of all varieties was lower than in the second week. In the second week, the Cytosolic APX gene was expressed in all treatments P0, P1, and P2, while the gene expression of all varieties in the fourth week was lower than in the second week. The *CAT* gene in the second week is highest in treatment P1 variety TN1, P0 variety Gogoniti II, and treatment P1 and P2 variety Cigeulis. In the fourth week, *CAT* gene expression was lower than in the second week, resulting in a faint line. The *GPOD* gene in the P1 and P2 treatments from the second week show higher expression than the P0 in all varieties. The *OsCATA* gene in the fourth week with P1 and P2 treatments of all varieties compared to P0. In the fourth week, the expression level of the *OsAPX* gene is lower than in the second week, and a faint line is also produced. In the second week, the OsCATA gene in the P1 and P2 treatments show higher expression than P0 in the varieties. In the fourth week, the OsCATA gene with P1 and P2 treatments showed higher expression in all varieties. The *OsCATA* gene in the fourth week with P1 and P2 treatments showed higher expression in all varieties than in the second week.

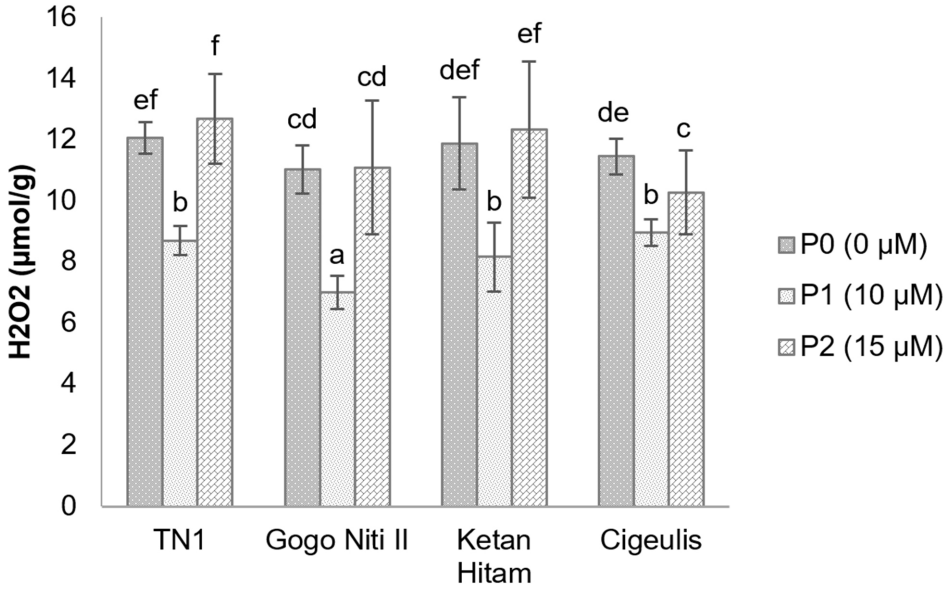
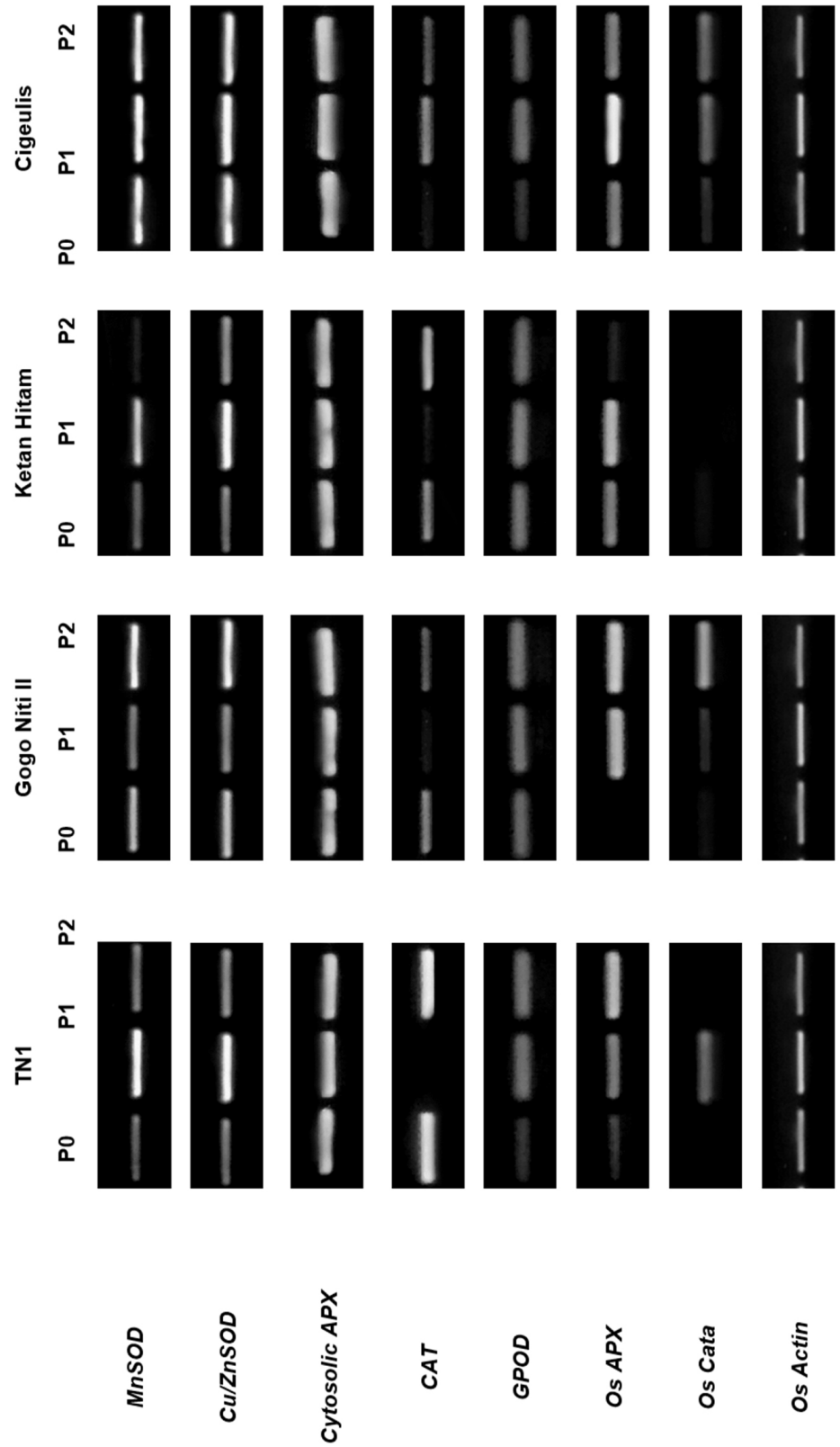
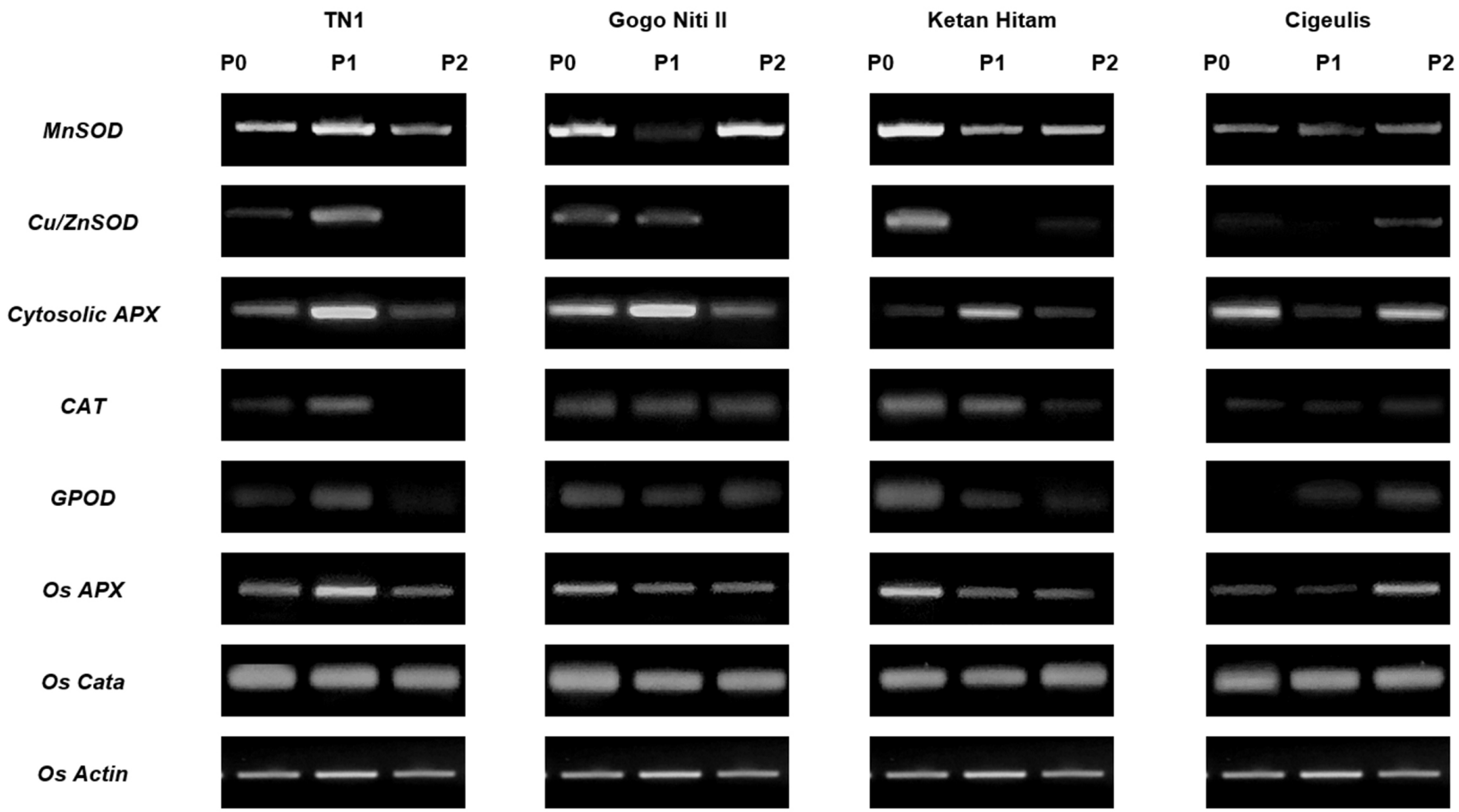


Fig. 9. Hydrogen peroxide content in all rice varieties treated with Melatonin in the fourth week. The difference in letters shows a significant effect through the DMRT test ($p \leq 0.05$; $n = 5$)



Remarks: P0: 0 µM Melatonin, P1: 10 µM Melatonin, P2: 15 µM Melatonin

Fig. 10. Electrophoresis results of antioxidant gene expression obtained from total RNA PCR analysis of callus samples on regeneration media with different hormones in the second week. OsACTIN is used as a housekeeping gene



Remarks: P0: 0 µM Melatonin, P1: 10 µM Melatonin, P2: 15 µM Melatonin

Fig. 11. Electrophoresis results of antioxidant gene expression obtained from total RNA PCR analysis of callus samples on regeneration media with different hormones in the fourth week. OsACTIN is used as a housekeeping gene

Table 4. Planlet morphology after 6 weeks in regeneration medium each variety

Variety	Treatment	Number of plantlets	Number of Leaves	Root length	Planlet Height
TN1	P0	2.33 ± 0.58 bc	1.33 ± 0.58	1.00 ± 0.00	2.33 ± 0.58
	P1	5.00 ± 1.00 a	1.67 ± 0.58	1.67 ± 0.58	4.00 ± 1.00
	P2	4.67 ± 0.58 a	1.33 ± 0.58	1.33 ± 0.58	3.00 ± 1.00
Gogo Niti II	P0	2.67 ± 0.58 bc	1.67 ± 0.58	1.00 ± 0.00	5.00 ± 1.00
	P1	5.67 ± 0.58 a	2.67 ± 0.58	2.00 ± 1.00	8.33 ± 1.53
	P2	3.00 ± 0.00 b	2.00 ± 0.00	1.33 ± 0.58	6.00 ± 1.00
Ketan Hitam	P0	2.00 ± 0.00 bc	1.00 ± 0.00	0.83 ± 0.29	2.00 ± 1.00
	P1	2.67 ± 0.58 bc	1.33 ± 0.58	1.67 ± 0.58	3.33 ± 0.58
	P2	1.67 ± 0.58 c	1.00 ± 0.00	1.00 ± 0.00	3.00 ± 1.00
Cigeulis	P0	3.00 ± 0.00 b	1.33 ± 0.58	0.83 ± 0.29	4.00 ± 1.00
	P1	5.33 ± 0.58 a	2.33 ± 0.58	1.67 ± 0.58	6.00 ± 1.00
	P2	2.33 ± 0.58 bc	1.67 ± 0.58	1.33 ± 0.58	4.33 ± 0.58

Remarks: The numbers followed by lowercase letters are the same in one column, meaning a significant difference in the DMRT mean difference test with a 95% confidence level

The high level expression of antioxidant genes such as *Cytosolic APX, OsAPX*, and *SOD* in the second week, as well as the high expression of OsCATA in the fourth week, indicate that the enzymatic antioxidant system activates them. The *CAT, APX*, and *SOD* genes are important antioxidant enzymes that play a part in increasing plant resistance to oxidative stress. The mechanism of the *SOD* enzyme itself is to form a line of defense against ROS, where it reduces superoxide radicals into H_2O_2. The *CAT* enzyme mechanism responds to H_2O_2 to catalyze the formation of H_2O_2 and O_2. Even the *APX* enzyme decomposes H_2O_2 into H_2, so it becomes a form harmless to plants by involving *GR, MDHAR, DHAR* in the *ASH/GSH* cycle (Das & Roychoudhury, 2014). Melatonin treatment's high antioxidant gene activity can influence the browning that happens. This is because Melatonin plays a role in removing phenolic compounds from tissue pieces that can cause browning. The phenolic compound can appear in the explants that failed to regenerate and then accumulate when there is an injury in explants to become browning (Anjarsari et al., 2022) However, adding Melatonin, the receptors will increase the content of Melatonin endogenous and then induce antioxidant genes such as *SOD, CAT, POD, GPX, APX*, or even *GSH* or *AsA* to become active and play a role in fighting accumulated ROS. (Pardo-Hernández et al., 2020).

Planlet Morphology

Based on Table 4 and Fig. 11, adding Melatonin using 10 µM (P1) concentration increased the number of plantlets, leaves, root length, and plantlet height. The Gogo Niti II variety with the 10 µM melatonin (P1) concentration had the best plantlet morphology through the parameters of the number of plantlets 5.00 ± 1.00 plantlets, number of leaves 2.67 ± 0.58, root length 2.00 ± 1.00 cm , and plantlet height 8.33 ± 1.53 cm.

The existence of the best plantlets indicates that the most appropriate dose to support growth and development from stress or cell death that occurs during the callus phase to regeneration is the addition of 10 µM melatonin (P1) to the regeneration medium with the best variety of Gogo Niti II. This is also supported by (Iqbal & Khan, 2022). In their study, melatonin can overcome stress problems and change cell metabolism if a stressor occurs.

CONCLUSION

Supplementation of 10 µM melatonin in a medium increased the morphogenesis of rice tissue culture. Exogenous Melatonin also showed expression level of antioxidant genes (*MnSOD, Cu/ZnSOD, Cytosolic APX, CAT, GPOD, OsAPX,* and *OsCATA*) in TN1, Gogo Niti II, Ketan Hitam, and Cigeulis varieties, higher than antioxidant gene expression in the fourth week during somatic

embryogenesis of rice. Further research can be done by adding levels of other melatonin concentrations to vary with other rice varieties because the genetic variation can make different responses and adding other supportive observational variables to describe Melatonin's role in scavenging reactive oxygen species.

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