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Full Length Article

The Effect of Melatonin on the Efficiency of Regeneration and Gene Expression during the Morphogenesis in Rice

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Abstract

Melatonin is a significant source of antioxidants and influences plant growth and development. In this research, we investigated the effect of different concentrations of melatonin on the efficiency of *in vitro* regeneration and gene expression during morphogenesis in rice (*Oryza sativa* L.). Data were recorded on the callus induction and regeneration phases to reveal the effects of melatonin treatments resulted in the highest rate of regeneration and gene expression in the varieties used. The results showed that 10 µM of melatonin promoted the regeneration frequency of Cigeulis (71%) and Ketan Hitam (68%) while 15 µM of melatonin promoted the regeneration frequency of TN1 (61%) and Gogo Niti II (76%). Based on PCR analysis, exogenous application of melatonin at 10 and 15 µM showed high expression of *OsSERK*, *OsLEC1* and *OsWOX4* genes than those without treatment, while *OsBBM* gene was not expressed in all treatments. Melatonin treatment during morphogenesis caused a positive response to generating planlets and gene expression.

Keywords: *In Vitro*; Gene expression; Melatonin; Morphogenesis; Rice

Introduction

Rice (*Oryza sativa* L.) is a monocotyledon plant and is an important food commodity for the community. This led to study the optimization of rice crop production needed. The application of tissue culture to rice plants is required to mass plant propagation in a shorter time and to develop biotechnology-based plants. The efficiency of *in vitro* plant regeneration greatly influences success in plant breeding efforts (Abiri *et al.* 2017). Indica and Javanica cultivars generally have lower regeneration ability than Japonica cultivars. Still, this reason cannot be used as absolute because the regeneration potential of rice plants does not always depend on the subspecies but also results from differences between the genotypes used (Akay and Kurt 2018).

Melatonin (N-acetyl-5-methoxytryptamine) is a neurotransmitter molecule in animals but is also involved in critical plant physiological processes such as regulating plant growth and development and increasing plant tolerance to stress. Melatonin also regulates gene expression and influences plant performance (Erland and Saxena 2018; Fan *et al.* 2018; Sharif *et al.* 2018). Nonetheless, the role of melatonin *in vitro* still needs to be better elucidated. The *in vitro* technique is interesting to study because it provides environmental conditions that can be explicitly controlled, making it easier to observe the processes that occur. The addition of low melatonin concentrations (<20 µM) increased shoot growth. In comparison, high concentrations (>20 µM) could reduce the growth effect or even have an inhibitory effect on rice plants (Liang *et al.* 2017). According to Ramakrishna *et al.* (2012), the addition of melatonin to tissue culture media can result in the formation of somatic embryos by changing the concentration of endogenous melatonin so that it can increase the induction of somatic embryogenesis.

The stages of plant morphogenesis are part of forming plantlets as a feature of cell totipotency. The process of plant morphogenesis through somatic embryogenesis means that a cell divides and undergoes differentiation to form an embryo. The development of somatic embryogenesis consists of an induction phase of somatic cells and an expression phase of embryogenic cells. After embryogenic induction is complete, monocot plants' next stages are the globular, scutellar, and coleoptile stages (Mastuti 2017). Somatic embryogenesis is regulated by the role of several

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genes, such as *SERK* (Somatic Embryogenesis Like Receptor Kinase), Leafy Cotyledon (*LEC*), Baby Boom (*BBM*), and Wuschel (*WUS*) (Gulzar *et al.* 2020). The *SERK* gene plays a role in forming embryogenic competence in the early stages of embryogenesis. *LEC* and *BBM* genes have almost the same function; namely, they play a role in the embryogenesis maturation phase, which supports the transition of embryogenic cells from non-embryogenic tissues. The *WUS* gene plays a role in the process of dedifferentiation of somatic cells followed by cell proliferation which can regulate somatic embryogenesis (Méndez-Hernández *et al.* 2019).

This study was aimed at to determine the effect of melatonin on regeneration efficiency and gene expression during morphogenesis in rice plants. In addition, the research results are expected to provide new knowledge about the role of melatonin in *in vitro* rice plant breeding.

Materials and Methods

Explant preparation

The explants were rice seed embryos peeled and sterilized using a 1% sodium chloride solution. Seed embryos were shaken using an orbital shaker at a speed of 120 rpm for 30 min and rinsed with sterile water three times and dried using filter paper.

Callus induction

The sterilized seeds were planted in the induction medium with the composition of the medium, including 4.14 g/L MS medium, 2 mg/L 2, 4-D, 30 g/L sucrose, and 4 g/L gelrite (Safitri *et al.* 2016). The pH of the solution was adjusted to 5.8 before autoclaving. The induction medium was sterilized in an autoclave at 121°C and 15 psi for 30 min. About 20–25 mL of medium was poured into the Petri dish under laminar airflow. The seeds were cultured on the induction medium under aseptic conditions and then incubated at 25 ± 2°C in the dark. The percentage of callus induction (Shahsavari *et al.* 2010) and callus size (Hoque *et al.* 2013) was observed three weeks after culture. Observations of callus morphology were carried out, including the callus color, structure and shape. Observation of callus morphology was carried out by microscopy in the third and eighth weeks on callus induction media.

$$\text{Percentage of callus induction} = \frac{\text{total number of callus}}{\text{number of explant}} \times 100\%$$

$$\text{Callus size} = \frac{(\text{width} + \text{length})}{2}$$

Plant regeneration

Eight weeks old embryogenic callus was sub-cultured to the composition of the regeneration medium following the procedure of Safitri *et al.* (2016) consisting of 4.41 g/L MS medium, 1 mg/L NAA, 2 mg/L Kinetin, 2 g/L casein hydrolysate, 30 g/L sucrose, 4 g/L Gelrite, and combined with melatonin (0, 10, 15 µM). Each treatment consisted of three replicates with 5 callus each. The callus was then incubated under 16 h photoperiod with light intensity 2000 lux at 24°C of room temperature. Plantlets regenerated *in vitro* were transferred to culture tubes containing the same regeneration medium for shoot elongation. *In vitro* regeneration response based on green spot formation was observed in the second and fourth weeks. In addition to the formation of green spots, observations were also made on the number of callus and callus formation that formed the globular, scutellar and coleoptile phases in the second- and fourth-weeks during plant regeneration. The number of growing plantlets was counted in the second week after transfer to the culture tube. The percentage of plant regeneration was calculated based on Karthikeyan *et al.* (2009).

$$\text{Green spot (\%)} = \frac{\text{Number of green spot on callus}}{\text{total number of explant}} \times 100$$

$$\text{Plant Regeneration (\%)} = \frac{\text{number of regenerated calluses}}{\text{number of induced calluses}} \times 100$$

Gene expression analysis

The gene expression observed was the *OsSERK*, *OsLEC1*, *OsWOX4*, and *OsBBM* genes (Table 1). Sampling of callus was carried out in the second and fourth weeks after culture on regeneration media. The stages in gene expression analysis were RNA isolation, cDNA synthesis, and PCR. Total RNA from the callus was extracted following the procedure Ribospin™ Plant Kit (GeneAll), and cDNA synthesis followed the procedure ReverTra Ace® qPCR RT Master Mix (Toyobo). Quantitative Polymerase chain reaction (Q-PCR) was performed with a total volume of 15 µL and following the GoTaq® Green Master Mix (Promega) procedure (Table 1). The amplified Q-PCR products were then electrophoresed in 2% agarose gel stained with EtBr and visualized using a UV transilluminator. The electrophoretic gel placed on the UV-transilluminator glowed orange from the formed DNA fragments. The DNA fragments were then documented and observed for the thickness of the bands.

Data analysis

Callus induction percentage and callus size were collected in the callus induction phase, while the percentage of green spots and regenerated plant were collected in the regeneration phase. The results were then analyzed using analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) was applied at a $P < 0.05$ to find significant differences among the treatments. Data obtained from gel electrophoresis visualization were analyzed using qualitative descriptive analysis with visual presentation.

Table 1: Primer sequences for gene expression analysis

Gene	Primer	NCBI Code
OsSERK	Forward: 5' TGC ATT GCA TAG CTT GAG GA 3' Reverse: 5' GCA GCA TTC CCA AGA TCA AC 3'	XM_015794373.2
OsWOX4	Forward: 5' CGC TAA CGA AAC CAA AGA GG 3' Reverse: 5' GGA AGA GCT CCA GGG TCA CT 3'	XM_015779881.2
OsLEC1	Forward: 5' CGT CGG TGG GAT GCT CAA GTC 3' Reverse: 5' GGT GCT CGA AGT TGA CGG TCT 3'	XM_015769434.2
OsBBM	Forward: 5' CGA TTT ACC GTG GCG TGA CA 3' Reverse: 5' CGT GAA GAG CAT CCT GGA CA 3'	XM_026019980.1
OsActin	Forward: 5' TCC ATC TTG GCA TCT CTC AG 3' Reverse: 5' GTA CCC GCA TCA GGC ATC TG 3'	XM_015774830.2

Table 2: Percentage of callus induction and its size

Rice variety	Percentage of callus induction (%)	Callus size (mm)
TN1	81.00 ± 5.37^{ab}	6.60 ± 0.31^{a}
Cigeulis	49.00 ± 5.22^{c}	4.93 ± 0.29^{b}
Ketan Hitam	70.20 ± 5.65^{b}	5.06 ± 0.19^{b}
Gogo Niti II	87.80 ± 2.31^{a}	6.76 ± 0.34^{a}

Note: numbers followed by the same letter show no significant difference in the 5% DMRT test.

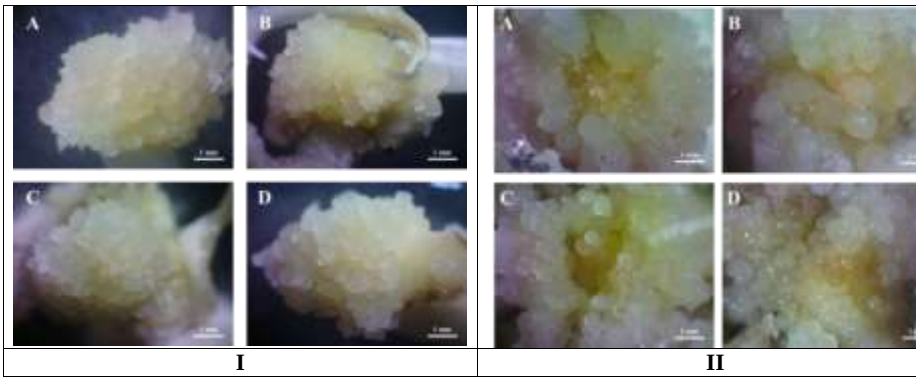

Fig. 1: Callus morphology of four rice varieties (A) TN1, (B) Cigeulis, (C) Ketan Hitam, and (D) Gogo Niti II after 3 weeks (**I**) and 8 weeks (**II**) on callus induction medium (scale bars = 1 mm)

Results

Callus induction

The use of 2, 4-D hormone of 2 mg/L in induction media significantly affected callus formation in each induced rice variety (Fig. 1). The percentage of callus induction was directly proportional to the size of the callus. Table 2 shows that the highest callus induction rate was in Gogo Niti II (87.80%), with a callus size of 5.06 mm, followed by TN1 (81%) with a callus size 6.60 mm, Ketan Hitam (70.20%) with a callus size of 5.06 mm, and the lowest one was in Cigeulis (49%) with the smallest callus size of 4.93 mm. During the induction process, the resulting callus was an embryonic callus that has the potential to develop into an embryo and plantlet. Data show the morphology of callus on rice varieties TN1, Cigeulis, Ketan Hitam, and Gogo Niti II for 4 and 8 weeks on induction media. The calluses of TN1, Cigeulis, Ketan Hitam, and Gogo Niti II in the third week were classified as type K2 (compact white) because all four had a compact structure and were white and had embryogenic potential. Meanwhile, the 8-week-old calluses of TN1, Cigeulis, Ketan Hitam, and Gogo Niti II can be classified in type K1 (greenish yellow) because they had a nodular structure, were brownish yellow and relatively soft (Fig. 1).

Regeneration

In the second week of observation, all varieties used, namely TN1, Cigeulis, Ketan Hitam, and Gogo Niti II, showed a significant increase in the percentage of green spots in the 10 and 15 µM melatonin treatments compared to the control (0 µM). In the fourth week, there was an increase in the percentage of green spots in all melatonin treatments, but not in the control treatment (Table 3.). After two weeks in the regeneration medium, the callus had developed into the globular and scutellar phases. All rice varieties in the second week entered the scutellar phase under 15 µM compared to 0 and 10 µM melatonin levels. Cigeulis produced the most callus that had entered the scutellar phase compared to other varieties under 15 µM

Table 3: Percentage of green spot and plant regeneration

Rice variety	Treatment (µM)	Percentage of green spot (%)		Plant regeneration (%)
		Second week	Fourth week	
TN1	0	41.67 ± 8.33^{b}	44.44 ± 5.56^{b}	0^{b}
	10	73.89 ± 3.89^{a}	73.89 ± 3.89^{a}	52.22 ± 7.78^{a}
	15	68.33 ± 9.28^{a}	76.67 ± 1.67^{a}	61.11 ± 5.56^{a}
Cigeulis	0	36.11 ± 7.35^{b}	38.89 ± 5.56^{b}	0^{c}
	10	78.33 ± 1.67^{a}	72.22 ± 2.78^{a}	71.11 ± 4.44^{a}
	15	65.56 ± 8.68^{a}	80.00 ± 0.00^{a}	38.33 ± 7.26^{b}
Ketan Hitam	0	44.44 ± 5.56^{b}	52.22 ± 7.78^{b}	0^{b}
	10	65.00 ± 12.58^{ab}	71.11 ± 4.44^{a}	68.33 ± 9.28^{ab}
	15	76.67 ± 1.67^{a}	78.33 ± 1.67^{a}	63.89 ± 7.35^{a}
Gogo Niti II	0	33.33 ± 8.33^{b}	36.11 ± 7.35^{b}	0^{c}
	10	60.00 ± 10.00^{ab}	69.45 ± 2.78^{a}	65.56 ± 8.68^{b}
	15	70.00 ± 10.00^{a}	76.67 ± 1.67^{a}	75.56 ± 4.44^{a}

Note: numbers followed by the same letter show no significant difference in the 5% DMRT test

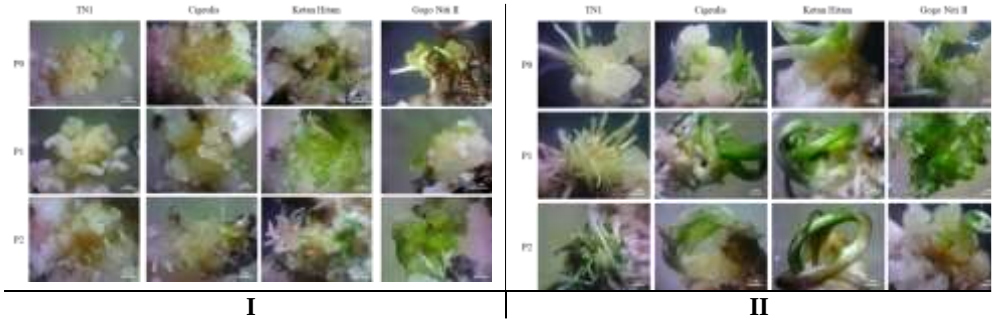

Fig. 2: Callus formation of four rice varieties in second week (**I**) and fourth week (**II**) after subculture to regeneration media with different melatonin concentrations. P0, P1 and P2 indicate 0, 10 and 15 µM melatonin treatments, respectively. Scale bars = 1 mm

melatonin with percentage of 33. Within four weeks, all rice varieties had entered the coleoptile phase with the most significant number at 15 µM compared to 0 and 10 µM treatments. The callus grown for six weeks on media without melatonin treatment did not produce plantlets because all callus experienced browning, while callus treated with 10 µM and 15 µM melatonin produced plantlets. The growth and development of plantlets were more towards shoot elongation than roots (Fig. 3).

Gene expression

Somatic embryogenesis gene expression was associated with developing somatic embryo morphogenesis in the second and fourth weeks (Fig. 4). Several embryogenesis genes expressed in callus regeneration treated with melatonin included the *OsSERK*, *OsLEC* and *OsWOX* (Fig. 4). In the second week, the *OsSERK* gene was expressed in all treatments, so this was associated with the role of the *SERK* gene in the development of the globular to scutellar phases. The *OsSERK* gene in the fourth week was expressed in all rice varieties treated with 0, 10, and 15 µM treatments. This is related to the formation of globular and scutellar phases in all rice varieties. The *OsLEC1* gene in the 10 and 15 µM treatments showed higher expression than 0 µM melatonin level in all rice varieties in the second week. The *OsWOX4* gene in all rice varieties treated with 15 µM melatonin was expressed higher than 0 and 1015 µM levels.

In the fourth week, the *OsWOX4* gene in TN1, Gogo Niti II, and Ketan Hitam with the 15 µM melatonin treatment showed higher expression (Fig. 4), which may be related to the callus that had entered the coleoptile phase more than under the 0 and 10 µM treatments. However, Cigeulis under 10 µM treatment showed higher expression of the *OsWOX4* gene, which may be related to the callus that had entered the coleoptile phase more than 0 and 15 µM (Fig. 4).

Discussion

The 2, 4-D is a synthetic auxin, which triggers most embryogenic callus growth in tissue culture systems for explant cell proliferation during the early stages of somatic embryo development (Loyola-Vargas and Ochoa-Alejo 2016). Callus proliferation response in rice varieties was different. Observation of the variable percentage of callus induction and callus size was made on induction media when the callus was three weeks old, which determined the potential for callus in each regenerated rice variety (Table 2). Callus induction was carried out for eight weeks with two subcultures. Calluses are classified into four types based on their morphological characteristics, including (1) "yellow/green" callus, a callus with a nodular structure that was greenish yellow and somewhat soft (K1), (2) "compact white" callus with smooth characteristics, white surface,

Table 4: The number of calli that entered the globular, scutellar and coleoptile phases

Rice variety	Treatment (µM)	Second week			Forth week		
		Globular	Scutellar	Coleoptile	Globular	Scutellar	Coleoptile
TN1	0	100 ± 0^{a}	0 ± 0^{b}	0	60 ± 0^{a}	40 ± 0^{a}	0^{c}
	10	80 ± 0^{b}	20 ± 0^{a}	0	40 ± 0^{b}	27 ± 7^{ab}	33 ± 7^{b}
	15	73 ± 7^{b}	27 ± 7^{a}	0	27 ± 7^{c}	13 ± 7^{b}	60 ± 0^{a}
Cigeulis	0	93 ± 7^{a}	7 ± 7^{b}	0	53 ± 7^{a}	47 ± 7^{a}	0^{b}
	10	80 ± 0^{ab}	20 ± 0^{ab}	0	33 ± 7^{ab}	27 ± 7^{ab}	40 ± 0^{a}
	15	67 ± 7^{b}	33 ± 7^{a}	0	27 ± 7^{b}	13 ± 7^{b}	60 ± 12^{a}
Ketan Hitam	0	100 ± 0^{a}	0^{b}	0	53 ± 7^{a}	40 ± 12^{a}	7 ± 7^{c}
	10	87 ± 7^{ab}	13 ± 7^{ab}	0	47 ± 7^{ab}	20 ± 0^{ab}	33 ± 7^{b}
	15	73 ± 7^{b}	27 ± 7^{a}	0	27 ± 7^{b}	7 ± 7^{b}	66 ± 7^{a}
Gogo Niti II	0	100 ± 0^{a}	0^{b}	0	53 ± 7^{a}	27 ± 7^{a}	20 ± 0^{b}
	10	87 ± 7^{b}	13 ± 7^{a}	0	40 ± 0^{ab}	20 ± 0^{ab}	40 ± 0^{ab}
	15	80 ± 0^{b}	20 ± 0^{a}	0	33 ± 7^{b}	7 ± 7^{b}	60 ± 0^{a}

Note: numbers followed by the same letter show no significant difference in the 5% DMRT test

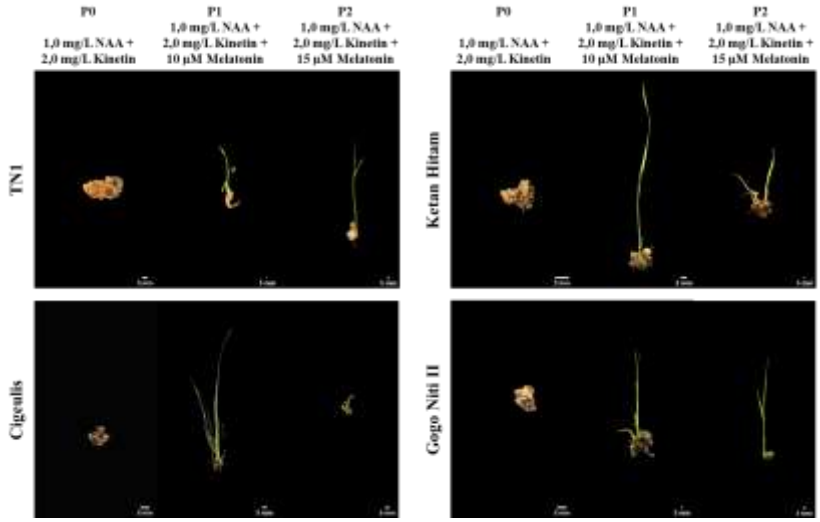

Fig. 3: Characteristics of regenerated plantlets from mature callus after the sixth week in regeneration media with different melatonin concentrations. P0, P1 and P2 indicate 0, 10 and 15 µM melatonin treatments, respectively. Scale bar = 10 mm

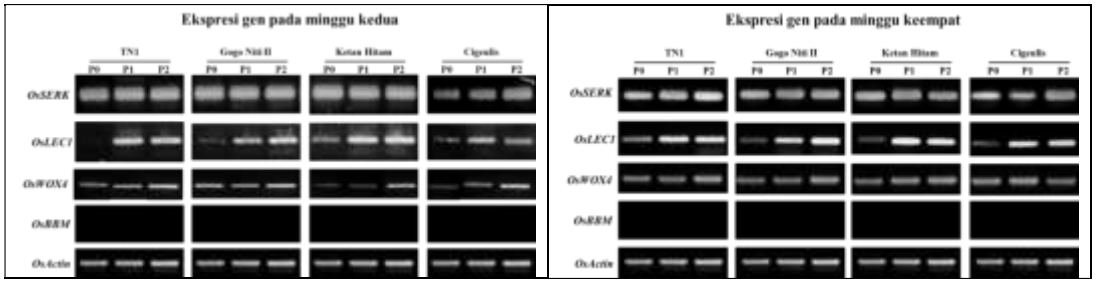

Fig. 4: Electrophoretic results of gene expression obtained from PCR analysis of total RNA of callus samples on regeneration media with different hormones in the second and fourth weeks. OsACTIN is used as a housekeeping gene. P0, P1 and P2 indicate 0, 10 and 15 µM melatonin treatments, respectively

generally has an embryogenic potential (K2), (3) "friable" callus, with a soft surface, looks watery, and low embryogenic potential, and (4) "browning" callus (K4) (Downey *et al.* 2019).

Mature callus resulting from long-term subculturing can reduce the embryogenic potential and decrease tissue quality, causing morphological changes in the callus (Quinga *et al.* 2017). This can be seen from the morphology of the 8-week-old callus, which began to turn brownish yellow and had a relatively soft texture. The selection of eight weeks old callus aims to determine the effect of melatonin administration on the regeneration efficiency of rice plants from mature callus.

The regenerated callus formed green spots due to

greening of callus when placed under irradiating light (Fig. 1; Table 3). The formation of green spots is an important phenomenon because it is used as an indicator of plant regeneration. Farhadi *et al.* (2017) stated that callus that turned to green continuously started shoot regeneration. The plant regeneration determines the number of plantlets that grow on the regenerated callus; the greater the number of green spots, the greater the potential to develop plantlets.

Characteristics of the callus that entered the globular, scutellar and coleoptile phases formed are shown in Table 4. The globular phase is characterized by a spherical shape that forms a scutellar phase embryo, a transitional phase into a coleoptile or the first growing young shoot (Zhao *et al.* 2017). The induced callus was still pro-embryonic. So, no visible regeneration progress had occurred. Ketan Hitam produced the callus that had entered the coleoptile phase as compared to other varieties in the 15 µM melatonin treatment with 66% success (Table 4).

The callus formations of TN1, Cigeulis, Ketan Hitam, and Gogo Niti II in the second and fourth weeks on regeneration media are shown in Fig. 2. Each rice variety developed a different morphology in response to melatonin treatment. In the fourth week, the callus showed specific characteristics of morphogenesis towards plant regeneration into plantlets. Characteristics of the regenerated plantlets are presented in Fig. 3. The absence of melatonin (0 µM) did not produce plantlets because all calli experienced browning. This can be caused by the synthesis of phenolic compounds, which can destroy callus cells and reduce the frequency of plant regeneration. Callus treated with melatonin can produce plantlets because melatonin has the ability to increase the photosynthetic efficiency of plants (Nawaz *et al.* 2021).

Both NAA and Kinetin stimulate cell division and improve somatic embryogenesis and plantlet regeneration (Mostafiz and Wagiran 2018). Melatonin increased regeneration in all varieties with the same induction and regeneration media treatment (Table 3). Melatonin carries out several plant functions, such as rhizogenesis, promotes plant growth, seed germination, and photosynthetic ability, and significantly acts as an antioxidant (Asif *et al.* 2019). Qiao *et al.* (2019) reported that the application of melatonin increased the wheat growth in N-deficient conditions.

In this study, the expression of the *OsSERK*, *OsLEC1*, *OsWOX4*, and *OsBBM* genes during somatic embryo development was analysed based on the thickness of DNA bands (Fig. 4). Thicker the band, the higher was the expressed gene. Gene expression analysis was not carried out in the early weeks of subculture on regeneration media, because the callus still did not show a response to somatic embryo development and there was still a 2, 4-D effect from the callus induction process. Therefore, this parameter focuses on the analysis of gene expression during the second and fourth weeks of somatic embryo development after the callus has differentiated on regeneration media. Fig. 4 shows that there were different gene expression patterns in each treatment.

The *SERK* gene and its role in somatic embryogenesis has been studied in many plant species (Ma *et al.* 2012; Porras-Murillo *et al.* 2018; Cueva-Agila *et al.* 2020). *SERK* gene expression was found in *Zea mays* during the five weeks culture period, which was closely related to the process of dedifferentiation and cell division at the stage of somatic embryo development in tissue culture systems (Zhang *et al.* 2011). The *SERK1* gene is also expressed along with the development of callus morphogenesis which shows embryogenic potential in *Ananas comosus* tissue culture (Ma *et al.* 2012). In addition, the expression level of *SERK1* in *Cedrela odorata* was higher in embryogenic callus than in non-embryogenic callus (Porras-Murillo *et al.* 2018). The *SERK* gene in *Cattleya maxima* also showed the highest expression level in the globular phase of the embryogenic callus (Cueva-Agila *et al.* 2020). The results of this study indicate that the application of melatonin in regeneration media may regulate the *SERK* gene (Fig. 4), which plays a role in the formation of embryogenic competence in the early stages of embryogenesis.

The *LEC* gene plays a role in the embryogenesis maturation phase, which was established during the second week in the 10 and 15 µM melatonin treatments to produce callus in the globular and scutellar phases the fastest in all rice varieties compared to 0 µM melatonin level. The *OsLEC1* gene in the fourth week with 10 and 15 µM melatonin treatment showed higher expression than in the second week. This indicated that the maturation phase of embryogenesis is increased, as indicated by number of calli that entered the scutellar and coleoptile phases. An increase in *LEC1* gene expression in embryogenic calluses until third week positively affected the maturation phase of *Medicago truncatula* embryos (Orłowska *et al.* 2017). The *LEC1* gene is also expressed during the development of somatic embryos from the globular phase to the heart-shaped phase in *Coffea canephora* (Nic-Can *et al.* 2013). The results of this study indicated that the application of melatonin in regeneration media can regulate the *LEC* gene (Fig. 4), which plays a role in the maturation phase of embryogenesis.

The *OsWOX4* gene expressed in all rice varieties treated with 15 µM melatonin in two weeks may associated with the role of the *WUS* gene in forming the scutellar phase which is the forerunner to forming coleoptile. The *WOX4* gene was also expressed between 0–21 days and was detected in the pro-embryonic phase of *C. canephora* in *in vitro* culture (Nic-Can *et al.* 2013). Other *WOX* gene family members are also found in several plant species, and their role in inducing the early stages of somatic embryo development and triggering embryonic cell regeneration in *Arabidopsis thaliana* (Haecker *et al.* 2004), *Vitis vinifera* (Dai *et al.* 2011), *Gossypium hirsutum* (Bouchabké-Coussa *et al.* 2013),

Populus trichocarpa (Kucukoglu *et al.* 2017), *M. truncatula* (Tvorogova *et al.* 2019), and *Glycine max* (Hao *et al*. 2019). This study revealed that the application of melatonin to regeneration media can regulate the *WUSCHEL* (*WUS*) gene, which plays a role in the dedifferentiation process when expressed in somatic cells followed by cell proliferation which can regulate somatic embryogenesis (Bouchabké-Coussa *et al.* 2013).

Regulation of somatic embryogenesis genes occurs in response to external stimuli such as hormones or certain stress conditions such as low or high temperatures, heavy metals, osmotic pressure, or drought stresses (Méndez-Hernández *et al.* 2019). Melatonin treatment and external stimuli may affect gene regulation during somatic embryogenesis. The *OsBBM* gene was not expressed in the second and fourth weeks, possibly due to melatonin treatment. This also occurred in *Larix decidua*, which showed low expression in the early stages of embryo development (8 and 19 days). The gene expression increased on day 34 during the embryonic maturation phase, namely cotyledon formation and hypocotyl elongation (Rupps *et al.* 2015).

The gene expression analysis results in the second and fourth weeks indicated that during somatic embryo development, epigenetic processes occurred showing a relationship between gene expression and morphological changes in plant regeneration in rice varieties and different melatonin treatments. This research has a novelty, namely melatonin treatment of 10 and 15 µM can regulate the expression of the *OsSERK*, *OsLEC1*, and *OsWOX4* genes during somatic embryo development in rice varieties TN1, Cigeulis, Ketan Hitam, and Gogo Niti II, as well as having a positive effect on the potential for morphogenesis by regenerating plantlets from callus cells.

Conclusion

Melatonin treatment efficiently improved the regeneration of rice *in vitro* and gene expression during morphogenesis. During the development of somatic embryos, epigenetic processes occur with the relationship between gene expression and morphological changes in plant regeneration. Mature callus regenerated in media with melatonin concentrations of 10 and 15 µM, suggests a better morphogenesis response than without melatonin treatment. Melatonin application also showed higher expression of *OsSERK*, *OsLEC1*, and *OsWOX4* genes in the rice genotypes in the second and fourth weeks during somatic embryo development. Meanwhile, the *OsBBM* gene was not expressed under melatonin treatment. This study provided new knowledge about the role of melatonin in *in vitro* rice breeding.

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Author Contributions

MU: conceptualized the study, interpreted the results and responsible for the content and similarity index of the manuscript. MU, NTH, ND interpreted the result. NAS and NNAA planned the experiments and the practical study. NAS, NNAA, MAM and NT wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this research.

References

Abiri R, M Maziah, NA Shaharuddin, ZNB Yusof (2017). Enhancing somatic embryogenesis of Malaysian rice cultivar MR219 using adjuvant materials in a high-efficiency protocol. *Intl J Environ Sci Technol* 14:1091–1108

Akay H, O Kurt (2018). Effects of Cultivar and explant sources on callus induction and plant regeneration in rice (*Oryza sativa* L.). *J Agric Sci Technol* 8:97–104

Asif M, A Pervez, R Ahmad (2019). Role of melatonin and plant-growth-promoting rhizobacteria in the growth and development of plants. *Clean Soil Air Water* 47:1800459

Bouchabké-Coussa O, M Obellianne, D Linderme, E Montes, A Maia-Grondard, F Vilaine, C Pannetier (2013). Wuschel overexpression promotes somatic embryogenesis and induces organogenesis in cotton (*Gossypium hirsutum* L.) tissues cultured *in vitro*. *Plant Cell Rep* 32:675–686

Cueva-Agila AY, N Alberca-Jaramillo, R Cella, L Concia (2020). Isolation, phylogenetic analysis, and expression of a Somatic Embryogenesis Receptor like Kinase (SERK) gene in *Cattleya maxima* Lindl. *Curr Plant Biol* 21:100139

Dai R, H Jin peng, Z Wang, P Avihai, H Xu Ying, W Zhang, S Chen wu, H Ma qin (2011). Cloning and characterization of wox4 gene from *Vitis vinifera* L. involved in stem cell regulation. *Agric Sci Chin* 10:1861–1871

Downey CD, J Zoń, AMP Jones (2019). Improving callus regeneration of Miscanthus × giganteus J.M.Greef, Deuter ex Hodk., Renvoize 'M161' callus by inhibition of the phenylpropanoid biosynthetic pathway. *In Vitro Cell Dev Biol – Plant* 55:109–120

Erland LAE, PK Saxena (2018). Melatonin in plant morphogenesis. *In Vitro Cell Dev Biol – Plant* 54:3–24

Fan J, Y Xie, Z Zhang, L Chen (2018). Melatonin: A multifunctional factor in plants. *Intl J Mol Sci* 19:1528

Farhadi N, J Panahandeh, AM Azar, SA Salte (2017). Effect of explant type, growth regulators and light intensity on callus induction and plant regeneration in four ecotypes of Persian shallot (*Allium hirtifolium*). *Sci Hortic* 218:80–86

Gulzar B, A Mujib, MQ Malik, R Sayeed, J Mamgain, B Ejaz (2020). Genes, proteins and other networks regulating somatic embryogenesis in plants. *J Genet Eng Biotechnol* 5:31

Haecker A, R Groß-Hardt, B Geiges, A Sarkar, H Breuninger, M Herrmann, T Laux (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development* 131:657–668

Hao Q, L Zhang, Y Yang, Z Shan, XA Zhou (2019). Genome-wide analysis of the wox gene family and function exploration of gmwox18 in soybean. *Plants* 8:215

Hoque KMA, ZA Azdi, SH Prodhan (2013). Development of callus initiation and regeneration system of different indigenous Indica rice varieties. *J Biol* 1:46–51

Karthikeyan A, S Thevar, K Pandian, M Ramesh (2009). High frequency plant regeneration from embryogenic callus of a popular Indica rice (*Oryza sativa* L.). *Physiol Mol Biol Plants* 15:371–375

Kucukoglu M, J Nilsson, B Zheng, S Chaabouni, O Nilsson (2017). WUSCHEL-RELATED HOMEOBOX4 (WOX4)-like genes regulate cambial cell division activity and secondary growth in Populus trees. *New Phytol* 215:642–657

Liang C, A Li, H Yu, W Li, C Liang, S Guo (2017). Melatonin regulates root architecture by modulating auxin response in rice. *Front Plant Sci* 8:134

Loyola-Vargas VM, N Ochoa-Alejo (2016). *Somatic Embryogenesis: Fundamental Aspects and Applications*. Springer, Cham, Switzerland

Ma J, Y He, C Wu, H Liu, Z Hu, G Sun (2012). Cloning and molecular characterization of a SERK gene transcriptionally induced during somatic embryogenesis in *Ananas comosus* cv. Shenwan. *Plant Mol Biol Rep* 30:195–203

Mastuti R (2017). *Dasar-Dasar Kultur Jaringan Tumbuhan*. Universitas Brawijaya Press, Kota Malang, Jawa Timur, Indonesia

Méndez-Hernández AH, M Ledezma-Rodríguez, RN Avilez-Montalvo, YL Juárez-Gómez, A Skeete, J Avilez-Montalvo, C De-la-Peña, VM Loyola-Vargas (2019). Signaling overview of plant somatic embryogenesis. *Front Plant Sci* 10:77

Mostafiz SB, A Wagiran (2018). Efficient callus induction and regeneration in selected indica rice. *Agronomy* 8:77

Nawaz K, R Chaudhary, A Sarwar, B Ahmad, A Gul, C Hano, BH Abbasi, S Anjum (2021). Melatonin as master regulator in plant growth, development and stress alleviator for sustainable agricultural production: Current status and future perspectives. *Sustainability* 13:294

Nic-Can GI, A López-Torres, F Barredo-Pool, K Wrobel, VM Loyola-Vargas, R Rojas-Herrera, C De-la-Peña (2013). New insights into somatic embryogenesis: LEAFY COTYLEDON1, BABY BOOM1 and WUSCHEL-RELATED HOMEOBOX4 are epigenetically regulated in *Coffea canephora*. *PLoS One* 8:e72160

Orłowska A, R Igielski, K Łagowska, E Kępczyńska (2017). Identification of LEC1, L1L and Polycomb Repressive Complex 2 genes and their expression during the induction phase of *Medicago truncatula* Gaertn. somatic embryogenesis. *Plant Cell Tiss Org Cult* 129:119–132

Porras-Murillo R, A Andrade-Torres, LY Solís-Ramos (2018). Expression analysis of two SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) genes during in vitro morphogenesis in Spanish cedar (*Cedrela odorata* L.). *3 Biotech* 8:470

Qiao Y, L Yin, B Wang, Q Ke, X Deng, S Wang (2019). Melatonin promotes plant growth by increasing nitrogen uptake and assimilation under nitrogen deficient condition in winter wheat. *Plant Physiol Biochem* 139:342–349

Quinga LAP, H Pacheco de Freitas Fraga, L do Nascimento Vieira, MP Guerra (2017). Epigenetics of long-term somatic embryogenesis in *Theobroma cacao* L.: DNA methylation and recovery of embryogenic potential. *Plant Cell Tiss Org Cult* 131:295–305

Ramakrishna A, P Giridhar, M Jobin, CS Paulose, GA Ravishankar (2012). Indoleamines and calcium enhance somatic embryogenesis in *Coffea canephora* P ex Fr. *Plant Cell Tiss Org Cult* 108:267–278

Rupps A, J Raschke, M Ru, K Zoglauer (2015). Identification of putative homologs of *Larix decidua* to BABYBOOM (BBM), LEAFY COTYLEDON1 (LEC1), WUSCHEL - related HOMEOBOX2 (WOX2) and SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK) during somatic embryogenesis. *Planta* 243:473–488

Safitri FA, M Ubaidillah, KM Kim (2016). Efficiency of transformation mediated by Agrobacterium tumefaciens using vacuum infiltration in rice (*Oryza sativa* L.). *J Plant Biotechnol* 43:66–75

Shahsavari E, AA Maheran, ASN Akmar, MM Hanafi (2010). The effect of plant growth regulators on optimization of tissue culture system in Malaysian upland rice. *Afr J Biotechnol* 9:2089–2094

Sharif R, CX Id, H Zhang, MBA Id, MA Id, QA Id, I Muhammad, A Shalmani, M Azher, N Id, P Chen, Y Li (2018). Melatonin and its effects on plant systems. *Molecules* 23:2352

Tvorogova VE, YA Fedorova, EA Potsenkovskaya, AA Kudriashov, EP Efremova, VA Kvitkovskaya, TW Wolabu, F Zhang, M Tadege, LA Lutova (2019). The WUSCHEL-related homeobox transcription factor MtWOX9-1 stimulates somatic embryogenesis in *Medicago truncatula*. *Plant Cell Tiss Org Cult* 138:517–527

Zhang S, X Liu, Y Lin, G Xie, F Fu, H Liu, J Wang, S Gao, H Lan, T Rong (2011). Characterization of a *ZmSERK* gene and its relationship to somatic embryogenesis in a maize culture. *Plant Cell Tiss Org Cult* 105:29–37

Zhao P, K Begcy, T Dresselhaus, MX Sun (2017). Does early embryogenesis in eudicots and monocots involve the same mechanism and molecular players? *Plant Physiol* 173:130–142