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The Study of Exogenous Auxin and Cytokinins in Embryogenesis and Fiber Genes Expression during *In Vitro* Regeneration of Cotton (*Gossypium hirsutum* L.)

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ABSTRACT

The efficiency of regeneration media, based on the morpho-cytochemical response and embryogenesis gene expression of widely cultivated cotton in Indonesia (*Gossypium hirsutum* L.), needs to be studied further. This study's objectives are to identify the effect of 2,4-D and IBA hormones on the morphology and induction of cotton callus; to determine the effect of regeneration media on the morphology, cytochemical, and gene expression of cotton plant during embryogenesis. This study was conducted by inducing cotton callus on MS0 medium, MS Basal + 0.1 mg/L 2,4-D and MS Basal + 0.1 mg/L IBA. The best callus induction results were regenerated on the regeneration media, namely M1 (MS Vitamin + 0.01 ppm 2,4-D + 0.3 ppm IBA + 0.5 ppm Kinetin), M2 (MS Vitamin + 0.01 ppm 2,4-D + 0.5 ppm IBA + 0.5 ppm Kinetin), M3 (MS Vitamin + 0.00 ppm 2,4-D + 0.3 ppm IBA + 0.5 ppm Kinetin), and M4 (MS Vitamin + 0, 00 ppm 2,4- D + 0.5 ppm IBA + 0.5 ppm Kinetin). The embryogenesis gene expression was observed. The best morphological results for callus induction were obtained on MS Basal + 0.1 mg/L 2,4-D medium with a compact and white callus, indicates high embryogenic and regeneration ability. The highest percentage of regenerated callus parameters was found in M2 media and further confirmed by cytochemical characterization. Embryogenesis genes expressed were GhSERK1, GhSERK2, LEC, GhWUS, and GhWOX11. Other fiber-related genes GhMYB25-like, GhHD-1, and F3H were also expressed during the embryogenesis.

1. Introduction

The composition of nutrients in the media is an important factor that needs to be considered in in vitro propagation. Each plant requires its own specific nutrients to grow optimally (Sudheer *et al.* 2022). The addition of exogenous hormones in tissue culture media can increase the regeneration efficiency. The high efficiency of regeneration media can be utilized for plant propagation using somatic cells (somatic embryogenesis) (Hesami *et al.* 2020). Somatic embryogenesis is a plant propagation with somatic cells in certain regeneration media, that are widely used as a tool for plant development through biotechnology. The implementation of plant biotechnology through somatic embryogenesis is used for genetic modification and character improvement in plant stock multiplication (Nic-Can *et al.* 2015).

Embryos produced in somatic embryogenesis originate from the differentiated somatic cells regulated by certain genes expressions. Several genes that are usually expressed in embryogenesis are WOX, WUS, SERK, baby boom (*BBM*), and *LEC* (Yang and Zhang 2010). Adding exogenous hormones to the regeneration media can cause different gene expression in plant regeneration. This condition is related to the presence of high levels of transcription in differentially expressed genes that promote cell differentiation and re-differentiation during callus induction and somatic embryogenesis (Liu *et al.* 2018; Xue *et al.* 2022).

Cotton (*Gossypium hirsutum* L.) is one of the potential plants in Indonesia that needs

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further studies for its embryogenesis genes. Somatic embryogenesis, as a tool in cotton plant improvement through genetic transformation, will provide information on cotton propagation efficiency, due to challenges of somaclonal variation, genotype-dependence, and lack of information on the regulation of embryogenesis genes involved in cotton embryogenesis (Kumar *et al.* 2015). In previous studies, several embryogenesis genes were confirmed to be expressed in several kinds of cotton in China and Brazil, such as *GhSERK1* (Shi *et al.* 2012; da Cunha Soares *et al.* 2018), *GhSERK2* (Liu *et al.* 2018), and *GhWUS* (Xiao *et al.* 2018). Wei *et al.* (2022) confirmed that auxin produced different expressions of the *GhWOX11* and *GhWOX12* genes, which controlled the formation of callus growth in cotton plants. Several other cotton embryogenesis genes were also expressed through somatic embryogenic multiplication in media with exogenous auxin and cytokinin (Wu *et al.* 2009).

In addition to its differentiation ability in the embryogenesis process, the development of cotton cell into fibers in mature plant through fiber-related gene expression in early stage of plant development needs to be confirmed. This is due to fiber as an expected product on cotton cultivation. Several genes have been verified to be involved in the following process, namely *GhMYB25-like* (Walford *et al.* 2011), *GhHD-1* (Walford *et al.* 2012), and *F3H* (Tan *et al.* 2013). This expression was confirmed through the early phase of fiber formation in the mature plant. Currently, there is no information regarding the expression of these genes during embryogenesis. Whereas, the information of fiber-related gene expression in the early stage of plant development is important in order to meet the most effective method on cotton improvement though biotechnology.

Biotechnology-based strategy for cotton fiber improvement is targeting genes for transformation that improve the conditions under which the fiber develops. The concept demonstrated potential of biotechnology applications on cotton fiber research and suggested new generations of fibers could be developed through genetic engineering in the early stage of embryogenesis. As embryogenesis and cotton fiber development involves many genes, it is important to understand the gene network and how the genes expressed influenced by exogenous plant hormones.

Exogenous auxin and cytokinin in culture media for regulating the expression of embryogenesis and fiber genes in mature plants have not been studied in many cultivated cotton plants in Indonesia. In fact, the exogenous auxin levels can affect the development of somatic cells caused by the activation of embryogenesis genes (da Chunha Soares *et al.* 2018). The regulation of embryogenesis genes can be a valuable resource regarding the cotton regeneration ability and information about the fiber-related genes expression, that can provide a powerful means to validate the fiber-related genes involved in the embryogenesis process. It is important to discover the information of embryogenesis and fiber-related genes expression, especially for the development of potential plants through a biotechnological approach. Therefore, this study was conducted to examine the effect of regeneration media on the expression of embryogenesis and fiber-related genes in *Gossypium hirsutum* L. var Kanesia 15 as the most cultivated cotton variety in Indonesia. This study aimed to identify the effect of 2,4-D and IBA hormones on cotton callus induction and determine morphology, cytochemical, and gene expression in cotton (*Gossypium hirsutum* L.) somatic embryogenesis on various regeneration media combinations.

2. Materials and Methods

This study used an experimental method conducted at the Agrotechnology Laboratory, University of Jember. The material used in this study consist of cotton seeds, MS (Murashige and Skoog) Basal media, MS (Murashige and Skoog) Vitamin media, and plant hormones such as 2,4-D, IBA, Kinetin, alchohol 96%, alchohol 70%, RPL buffer, RBW buffer, RNW buffer, primer, nuclease-free water, liquid nitrogen. The experimental design used in this study was a Completely Randomized Design. For callus induction, three treatments were applied, namely control, MS Basal with 0.1 mg/L 2,4-D, and MS Basal with 0.1 mg/L IBA. Each treatment had 10 replications. Meanwhile, plant regeneration was composed of 4 treatments with six replications.

2.1. Explant Preparation

Cotton seeds (*Gossypium hirsutum* L.) were sterilized in 10% chlorox solution for 8 minutes and rinsed using a sterile distilled water at three times. The sterile cotton seeds were then planted on MS Basal media.

The explants used in this study were hypocotyl from cotton seeds grown on MS0 media.

2.2. Callus Induction

Explant for callus induction was harvested from the germinated cotton seeds hypocotyl planted on MS Basal media at the age of 7 to 14 DAP (days after planting). Cotton hypocotyl were divided into a small part to induce the callus by the thin layer culture method. Calluses were induced for four weeks in several induction such as MS0 media (MS media without any additional exogenous hormone) as the control, MS media with 0.1 mg/L 2,4-D MS media with 0.1 mg/L IBA. The calculation of callus induction calculated by the following formula based on Shahsavari *et al.* (2010) and Haryadi *et al.* (2023) .

$$\text{Callus induction (\%)} = \frac{\text{Number of calli}}{\text{Number of cultured explants}} \times 100\%$$

The percentage of callus induction and callus diameter were observed every two weeks. Callus morphology, including the callus structure, shape, and color, was visually observed using a microscope. Callus morphology was observed, following the callus characterization of Downey *et al.* (2019).

2.3. Plant Regeneration

The best callus induction results were regenerated on the regeneration media with different combinations of auxin and cytokinin hormones as explained in Table 1. The callus was incubated in a growth chamber under the 16/8 photoperiod with light intensity of 2,000 lux at 24°C of room temperature, then sub-cultured every two weeks in the regeneration media. C allus was regenerated for 10 weeks to obtain cotton planlet. Regeneration response based on the morphological characters, i.e. the green spot formation and globular percentage, were observed on 2 WAT (weeks after treatment), and coleoptile was observed in 4 WAT, following Haryadi *et al.* (2023). The cotton planlet obtained at 10 WAT (week after treatment) were transferred to MS0 media for 2 weeks to determine the number of plantlets obtained. The cytochemical characterization of cotton callus was carried out, following Hui-Hui *et al.* (2019). The cotton calli from each treatment were taken and stained with 2% Acetocarmine for 2 minutes, then washed with sterile distilled water three times. The callus was stained using the Evans Blue 0.5% for 30 seconds and washed to remove the remaining dye. The samples were observed under the microscope.

Table 1. Concentration of regeneration media

Treatment	Kind of MS media	Concentration of hormones (ppm)		
		2,4-D	IBA	Kinetin
M1	MS Vitamin	0.01	0.3	0.5
M2	MS Vitamin	0.01	0.5	0.5
M3	MS Vitamin	0.00	0.3	0.5
M4	MS Vitamin	0.00	0.5	0.5

2.4. Gene Expression Analysis

For somatic embryogenesis-related genes, *GhSERK1*, *GhSEERK2*, *GhWUS*, *GhWOX11*, *LEC*, and *BBM* were observed, while *GhMyB25-like*, *F3H*, *GhHD1* were observed as the fiber-related genes (Table 2). Callus was sampled on 2 and 4 WAT in the regeneration media. The expressions of *GhSERK1*, *GhSERK2*, *GhWOX11*, *GhWUS*, *BBM*, and *LEC* as embryogenesis related genes were analyzed to determine cotton embryogenesis gene activity response in different regeneration media. The *GhMYB25-like*, *GhHD-1*, and *F3H* expressions were analyzed to identify, whether these fiber-related genes were expressed during embryogenesis.

Gene expression analysis stages consist of RNA isolation, cDNA synthesis, and PCR. Total RNA was extracted following the Ribospin™ Plant Kit procedure (GeneAll), and cDNA synthesis followed the ReverTra Ace® qPCR RT Master Mix procedure (Toyobo). The Quantitative Polymerase chain reaction (Q-PCR) was performed with a total volume of 15 µL, following the GoTaq® Green Master Mix (Promega) procedure. The amplified Q-PCR products were then electrophoresed in 2% agarose gel, stained with EtBr, and visualized using a UV transilluminator. The electrophoretic gel was placed on the UV-transilluminator with an orange-colored glow from the formed DNA fragments. The DNA fragments were then documented and observed for the band thickness.

2.5. Data Analysis

The callus induction stage data were analyzed using an Analysis of Variance (ANOVA). Significantly different data foundafter the ANOVA analysis were further analyzed using the Tukey post-hoc test to determine the best treatment factor in callus formation. In plant regeneration stage, the

Table 2. Primer sequence for gene expression analysis

Gene	Primer	Source
GhSERK1	F 5′ GCATGATCATTGTAACCCCAAG 3′ R 5′ GGTATTCAGGGGCTATATGACC 3′	(da Cunha Soares *et al.* 2018)
GhSERK2	F 5′ CGGTTATGGTGTTATGCTTCT 3’ R 5’GCTCCACTTCTTCGTCTATGT 3’	(Liu *et al.* 2018)
GhWOX11	F 5’ AAAACCGGCGTTCTAGGTCTCG 3’ R 5’ GTATACTGCTTGGGCTTGGGGG 3’	(Wei *et al.* 2022)
GhWUS	F 5’ CTGATATTCTCCCCATGCAAACA 3’ R 5’ CGAGCAATCCCTAAAACTCTTCT 3’	(Xiao *et al.* 2018)
OsLEC	F 5’ CGT CGG TGG GAT GCT CAA GTC 3’ R 5’ GGT GCT CGA AGT TGA CGG TCT 3’	(Haryadi *et al.* 2023)
OsBBM	F 5’ CGA TTT ACC GTG GCG TGA CA 3’ R 5’CGT GAA GAG CAT CCT GGA CA 3’	(Adnan *et al.* 2022)
OsActin	F 5’ TCC ATC TTG GCA TCT CTC AG 3’ R 5’ GTA CCC GCA TCA GGC ATC TG 3’	(Haryadi *et al.* 2023)
F3H	F 5’ GGGCCTAGCTTGCAAGCTTCTT 3’	Tan *et al.* (2013)
GhHD-1	F 5’ GCT GAA GTT GTT GGA TGT GTC TTT 3’	Kim *et al.* (2015)
GhMyb25-like	F 5’ GAG AAA TCG AGC CAA GTT GC 3’	Kim *et al.* (2015)

observed data were analyzed using an Analysis of Variance (ANOVA). Significantly different data occurred after the ANOVA analysis were then analyzed using the DMRT (Duncan's Multiple Range Test). Statistical Package for Social Sciences (SPSS) software for Windows version 16.0 was used to analyze the quantitave data. The expression of somatic embryogenesis and fiber-related genes were analyzed using qualitative descriptive analysis by presenting the visual data.

3. Results

3.1. Callus Induction

Analysis of Variance result showed a significant different on callus diameter size (Table 3). Cotton callus induction on three different induction media were categorized into callus size and callus morphology. The use of MS basal media with different auxin hormones, namely IBA and 2,4-D, could induce the cotton callus from hypocotyl explants with the best average callus size was obtained from the Basal MS media + 0.1 mg/L 2,4-D (Table 3).

Based on the visual observation, the 2-WAT callus induction through hypocotyl explants showed no callus formation. At 4 WAT, the morphological characters of the callus on 2,4-D hormone media obtained a white callus color. In contrast, the callus from the induction media with IBA hormone yellowish-white (Figure 1). Based on the following conditions, there are differences in callus morphological characters in terms of size, color, and callus texture. Morphologically, calli on 2,4-D media had a high ability to regenerate better than the IBA media (Table 4). The embryogenic callus induction in cotton cultivation is required as a basis for the best induction media selection, before the callus is regenerated. The best embryogenic callus was obtained from the MS medium + 0.1 mg/L 2,4-D. Therefore, calli from MS media with 0.1 mg/L 2,4-D were chosen to be regenerated further on the regeneration media with different combinations of auxin and cytokinin hormones.

3.2. Morpho-cytochemical Character on Plant Regeneration

Figure 2 showed the biggest callus size that was found in the M2 regeneration medium (MS Vitamin + 0.01 mg/L 2,4-D + 0.5 mg/L IBA + 0.5 mg/L Kinetin). The various result on morphological response indicated by the greenspot, globular, and coleoptile were obtained on different media (Figure 3). Likewise, the cytochemical finding (Figure 4) showed the different responses in the callus regeneration process across various media. In the second week, the regenerated callus on all regeneration media showed green spots and entered a globular phase as one of the regeneration process characteristics (Figure 5A). The number of calli entering the coleoptile phase was counted in the fourth week, but at 2 WAT, calli on the M2 medium has started to enter the coleoptile phase (Figure 5B). The best percentage of coleoptile was obtained from the M1 medium, but not significantly different from the M2 medium (Table 5).

Table 3. Percentage of callus induction, callus weight and callus diameter. Data were analyzed using ANOVA and significant results were analyzed using the DMRT with 95% level of confidence

Media	Callus induction (%)	Callus weight (mg)	Callus diameter (mm)	
			2 WAT	4 WAT
MS0	0	-	-	-
MS Basal + 0.1 mg/L IBA	100	4.8±1.31	1.26±0.11[a]	1.66±0.17[a]
MS Basal + 0.1 mg/L 2,4-D	100	5.9±3.68	1.31±0.12[b]	2.03±0.07[b]

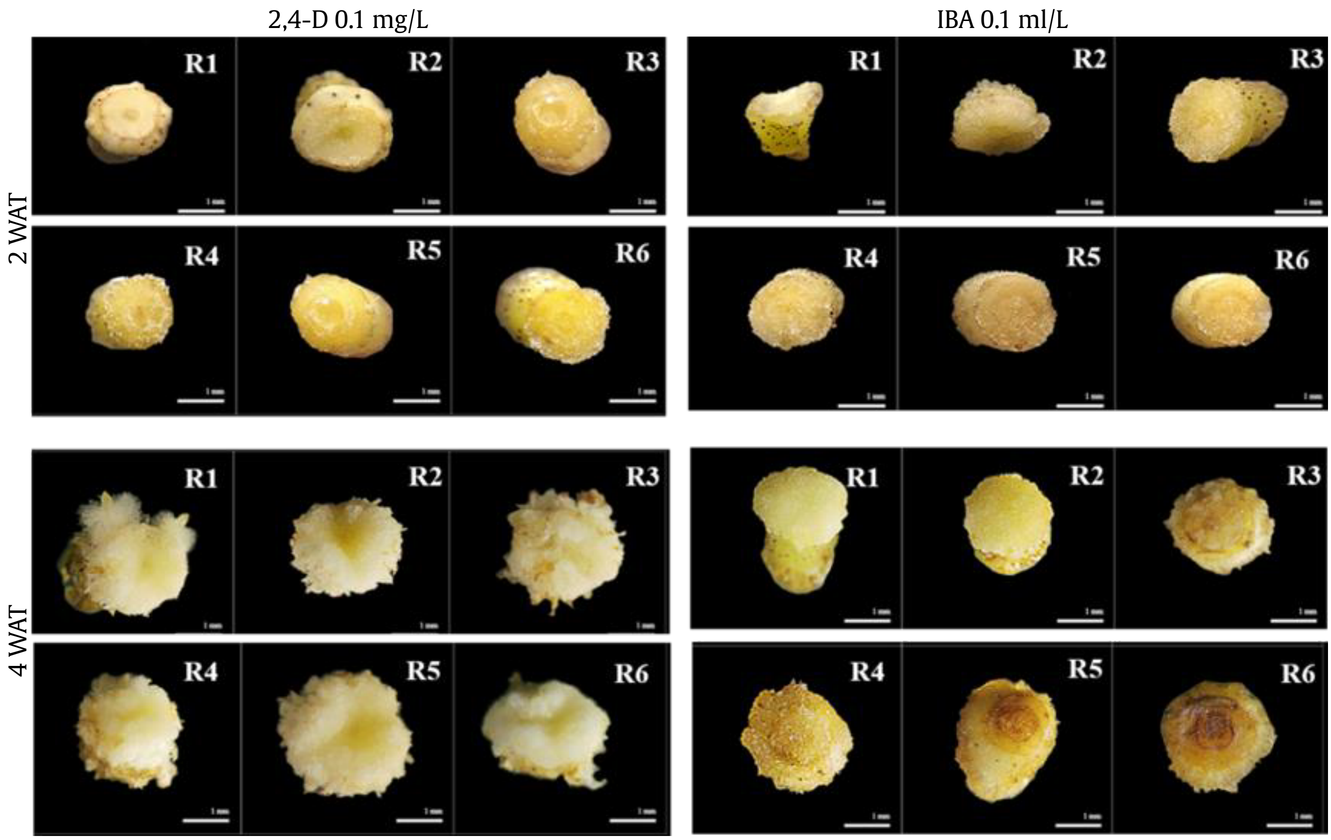

Figure 1. The morphological character of cotton calli. Scale bars: 1 mm, WAT: weeks after treatment, R: replication

Table 4. Callus morphology

Media	Callus texture	Callus color	Category	Embryogenic/ regeneration ability
MS0	-	-	-	
MS Basal + 0.1 mg/L 2,4-D	Compact	White	K2	HER
MS Basal + 0.1 mg/L IBA	Fragile	White-yellowish	K3	HENR

HER (High Embryogenic and Regeneration Ability); HENR (High Embryogenic but No Regeneration Ability); K2: compact white callus; K3: fragile callus (Downey *et al.* 2019)

Based on morphological characteristics, 2,4-D, IBA, and Kinetin combinations on the regeneration media (M1 and M2) showed the best regeneration result by producing shoots at 10 WAT as an indicator of planlet formation (Figure 3). The highest number of shoots was found in M2 regeneration medium at 50%. The best number of cotton plantlets (Figure 6) was obtained from the cotton regenerated in M2 medium (Table 5) at 50%. Meanwhile, calli regenerated on M3 and M4 media has turned to more browning color, without any plantlets. The best results in the M2 and M1 media were also confirmed by the cytochemical analysis with Acetocarmine as a dominant red stain, which means that there are more embryogenic cells.

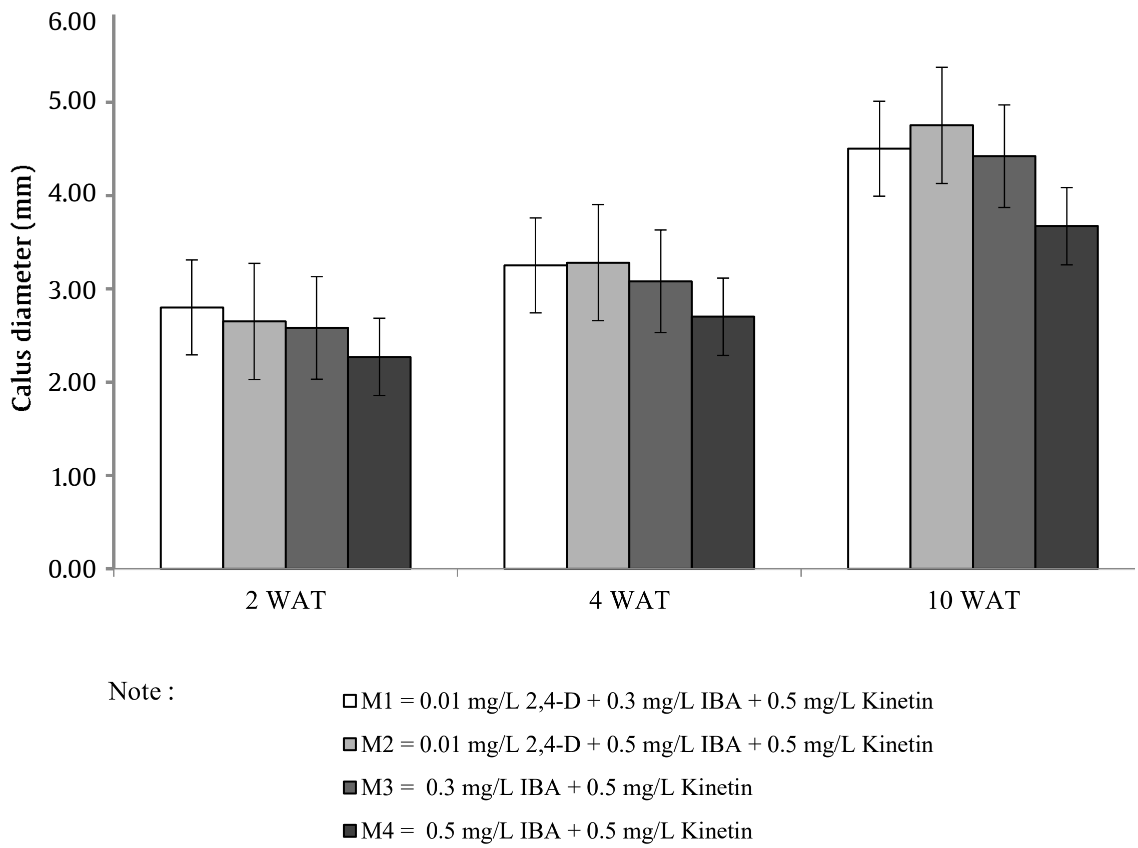

Figure 2. Callus size on the regeneration phase. The ANOVA test showed non-significant results in all treatments. WAT: weeks after treatment

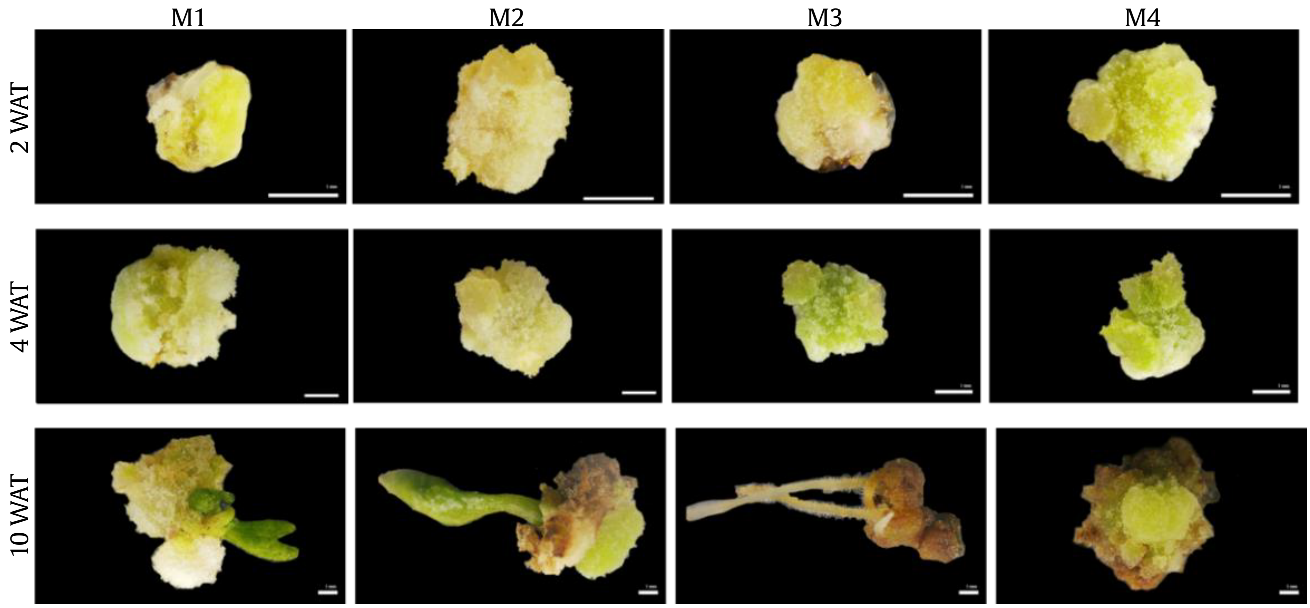

Figure 3. The Cotton Callus Morphology on The Regeneration Phase. Scale bars: 1 mm, WAT: weeks after treatment, M: regeneration media

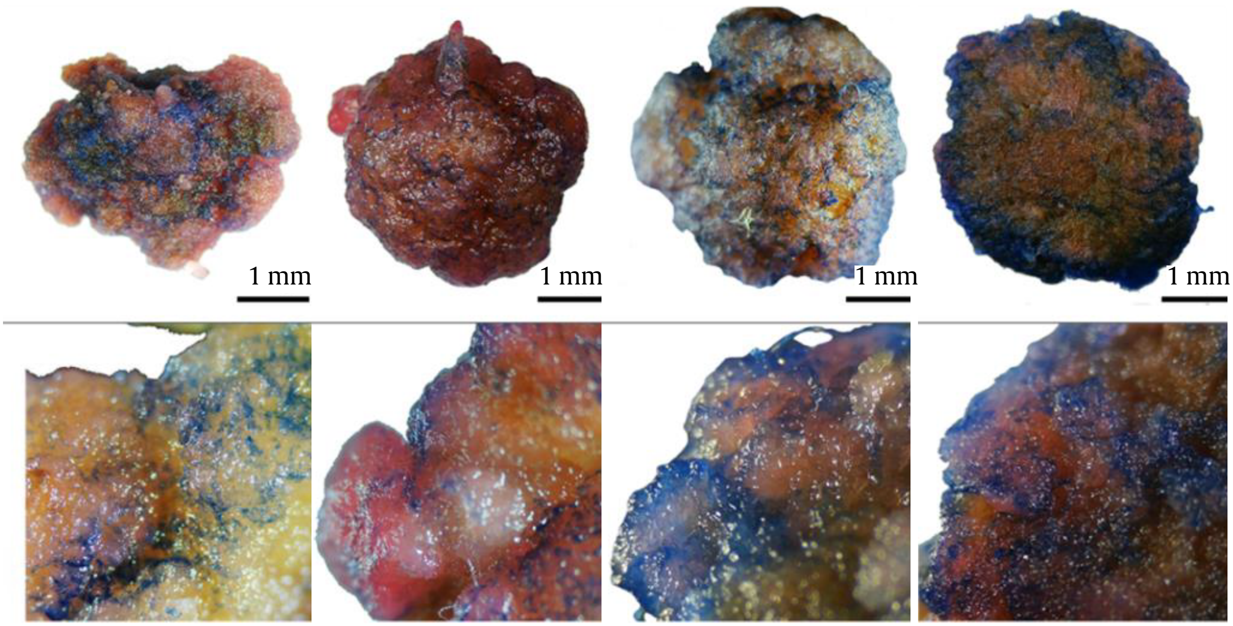

Figure 4. Cytochemical character of cultured cotton cell. Embryogenic cells are stained red, non embryogenic cells are stained blue. The red color indicates that the cells have more embryogenic potential, scale bars: 1 mm, M: regeneration media

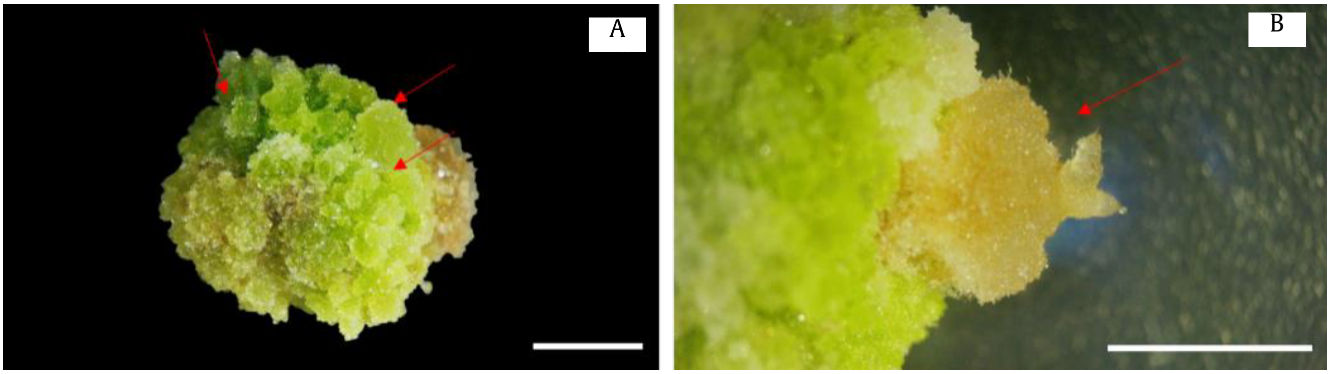

Figure 5. Globular (A) and coleoptile (B) formation. Red arrows point the parts of the calli on globular phase (A) and coleoptile (B). Scale bars: 1 mm

Table 5. Green spot, globular, coleoptile, and plant regeneration percentage. Data were analyzed using ANOVA and significant results were analyzed using DMRT post-hoc test with 95% level of confidence

Media	Green spot (%)	Globular (%)	Coleoptile (%)	Regenerated callus (%)
M1	100±0.00	100±0.00	25.00±9.13^{c}	33.33±10.53^{c}
M2	100±0.00	100±0.00	16.67±10.54^{bc}	50.00±14.91^{d}
M3	100±0.00	100±0.00	0.00±0.00^{a}	0.00±0.00^{ab}
M4	100±0.00	100±0.00	16.67±0.00^{b}	0.00±0.00^{a}

In contrast, the M3 and M4 media showed, that the culture cells were dominated by the absorption of blue stain that indicating the membrane ruptures. The double staining method can be used to confirm the embryogenic potential by distinguishing the embryogenic cell and non-embryogenic cell through double staining. Through cytochemical analyses, embryogenic-cell absorbed the red stain, while non-embryogenic cells absorbed the blue stain.

3.3. Gene Expression

Based on the visualization using electrophoresis (Figures 7 and 8), several embryogenesis related genes were expressed in various regeneration media

Figure 6. Cotton planlets on 12 WAT (weeks after treatment)

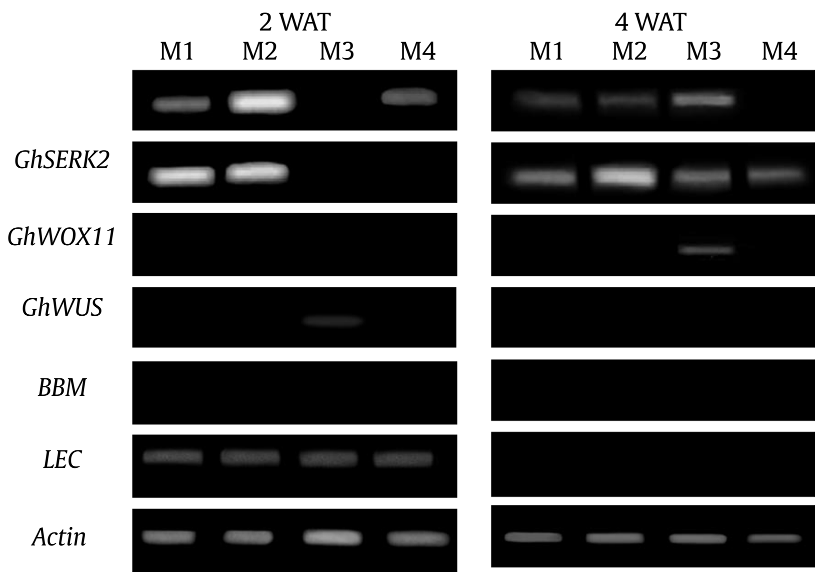

Figure 7. Visualization of embryogenic gene expression in cotton (*Gossypium hirsutum* L.). M: regeneration media

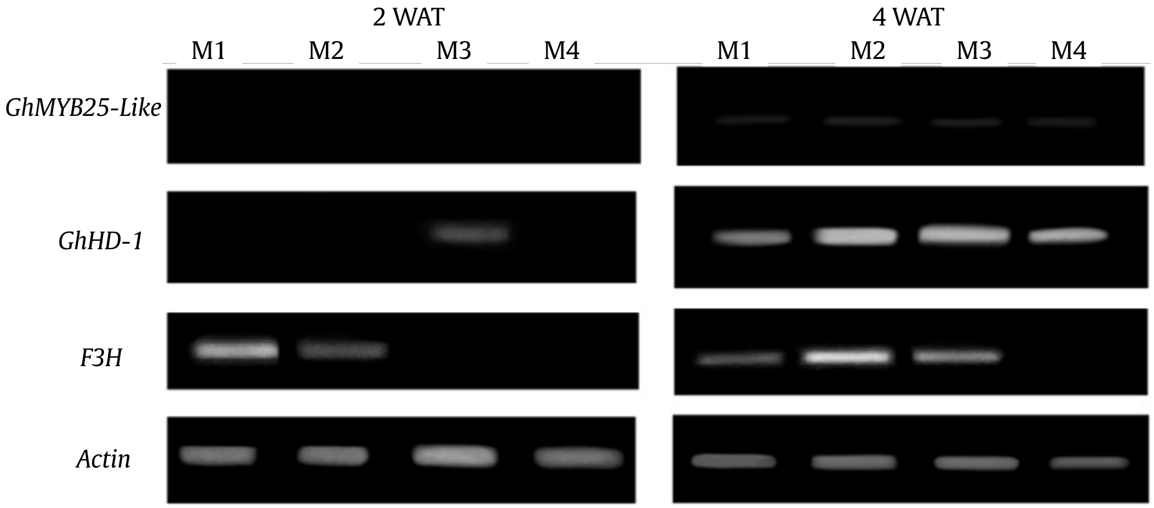

Figure 8. Visualization of fiber-related gene expression in cotton (*Gossypium hirsutum* L.). M: regeneration media

treatments, both at 2 WAT and 4 WAT. Among the observed genes, the BBM gene was unexpressed in all treatments at 2 WAT and 4 WAT. In addition, the *GhWOX11* and *GhWUS* genes were only expressed on the M3 regeneration medium. The highest expression of embryogenesis genes was found in the M1 and M2 regeneration media. The *GhSERK1* and *GhSERK2* genes expression level in the M2 regeneration medium showed a higher expression level than the M1 media. Moreover, the fiber-related genes showed various expressions. At 2 WAT, the expression of *GhMYB25-like* in all media treatments was still unexpressed, yet the genes were all expressed in all treatments at 4 WAT. The *GhHD-1* expression in 2 WAT was only found in the M3 medium, whereas there was an increased gene expression on the M1, M2, and M3 media treatments with the highest expression level in the M2. The *F3H* in the 2 WAT was expressed in M1 and M2 media, whereas *F3H* was still expressed in the same treatment at 4 WAT, followed by the expression in M3 media. Based on the results, only *F3H* was identified as a directly proportional to the best embryogenesis process in the M2 and M1 media.

4. Discussion

Callus induction was carried out as a way to obtain the cell differentiation to become an embryo. Callus formation is highly dependent on the availability and amount of exogenous auxin in the media (Rasud and Bustaman 2020). In this study, the presence of exogenous auxin, namely 2,4-D, obtained the best callus morphology and induction results. The success of callus induction on 2,4-D media was also confirmed in *Aerides odorata* Lour. (Khalida *et al.* 2019), *Barringtonia racemosa* (Dalila *et al.* 2013). The exogenous auxin was applied to cotton plants for inducing the callus on 2,4-D, while IBA showed a medium ability on callus induction (Hui-hui *et al.* 2019). In this case, the addition of IBA hormone in inducing the cotton callus should no better than 2,4-D. The use of IBA in callus formation in cell dedifferentiation has also not been widely applied for the organogenesis of root formation (Babashpour-Asl 2012; Fattorini *et al.* 2017; Justamante *et al.* 2022).

The presence of auxin in plant tissues affects the callus formation and morphology (Bano *et al.* 2022). Based on the morphological characters, there are two types of callus characters obtained from the use of 2,4-D and IBA hormones for callus induction. The K2 callus category was obtained on the 2,4-D media and the K3 callus category was obtained on the IBA media. The callus induction in 2,4-D media showed embryogenic callus characteristics with high ability to regenerate, according to Downey *et al.* (2019). The visual appearance of callus morphology through the color condition can indicate the cell division activity that occurs in the callus. The white, light yellow, or yellow-colored calli indicate that the cell division is still occurring, while brown callus indicates that the callus cells are aging (Astutik *et al.* 2021). The calli on 2,4-D media have a compact shape with white-colored condition, which indicate a meristematic tissue in the callus (Klimek-Chodacka *et al.* 2020). In this study, the 2,4-D hormone could induce the cotton calluses with the best results, but the use of 2,4-D concentrations needs further confirmation in future studies.

Several plant development phases characterize the regeneration stages in somatic embryogenesis. The embryogenic callus regeneration on four different media combinations showed significant coleoptile and plant regeneration data. In this study, green spots were observed at 2 WAT in all regeneration media (Table 5). The presence of green spots indicates that the regenerated callus is embryogenic (Noor *et al.* 2022). Apart from the green spot, the globular phase (Figure 3A) was observed in all regeneration media, and the coleoptile phase was observed in the M2 media at 2 WAT (Figure 3B). The hormone types greatly influences the direction of cotton plants regeneration, which are propagated *in vitro*.

The role of auxin and cytokinins in the cotton callus regeneration stage determines the direction of cotton regeneration. Auxins and cytokinins have antagonistic roles in the plant differentiation phase, but both are mutually dependent on one another (Chandler and Werr 2015). In this study, the combination of 2,4-D media, IBA, and kinetin at different concentrations (M1 and M2) resulted in the cotton regeneration to become plantlets. The combination of 2,4-D, IBA, and kinetin in the regeneration media with 0.1 mg/L kinetin was also confirmed to produce the best somatic embryos in *Gossypium arboreum* (Ke *et al.* 2021).

At the molecular level, plant regeneration through somatic embryogenesis is inseparable from the regulation of embryogenesis-related genes as one of the regeneration parameters. In this study, the presence of exogenous auxin and cytokinin at the different level of concentration affect the result of gene expression during somatic embryogenesis. Auxin has been confirmed to be a factor that involved with the up-regulation of cell-signaling genes (Pandey and Chaudary 2014).

The *GhSERK1* gene was expressed at 2 and 4 WAT in the M1 and M2 regeneration media, but it was only expressed in M4 at 2 WAT, then in M3 at 4 WAT. In this study, the expression of *GhSERK1* in M1 and M2 regeneration media was directly proportional to the desired regeneration results. The *GhSERK1* positive regulation in cotton plants occurs in embryogenic callus (da Cunha Soares *et al.* 2018). The positive regulation of SERK in embryogenic callus was also confirmed in *Araucaria angustifolia, Trifolium nigrescent*, and *Cattleya maxima* (Steiner *et al.* 2012; Pilarska *et al.* 2016; Cueva-Agila *et al.* 2020). *GhSERK1* has a broad role in the aspect of cotton plant development.

Besides *GhSERK1*, the *GhSERK2* also belongs to the functional SERK family and is associated with somatic and zygotic embryogenesis (Liu *et al.* 2018). In the cotton regeneration, the *GhSERK2* was only expressed in the M1 and M2 regeneration media at 2 WAT. However, the *GhSERK2* was expressed in all regeneration media with different expression levels at 4 WAT. Different auxin hormone content at the same kinetin level in the regeneration medium affected the expression period of the *GhSERK2* gene. In this study, high levels of SERK gene expression occurred in the globular phase. High expression of *GhSERK2* gene was also identified during the globular phase in *Brassica napus, Cocos nucifera, Momordica charantia* (Talapatra *et al.* 2014; Ahmadi *et al.* 2016; Rajesh *et al.* 2016). The SERK gene expression is up-regulated by the presence of auxins and cytokinins in plants (Porras-Murillo *et al.* 2018). High expression level *GhSERK2* gene is triggered by the 2,4-D hormone during the early phases of somatic embryogenesis in cotton plants (Liu *et al.* 2018).

BBM (*THE BABY BOOM*) is included in the transcription factor of several genesandhas an essential role in the embryogenesis and proliferation process. in this study, the expression of *BBM* was unconfirmed either at 2 WAT or 4 WAT regeneration. This absence indicates that there is no activity of the BBM gene in the observed phase. In other studies, the expression of BBM gene in *Arabidopsis thaliana* (Lutz *et al.* 2015), *Glycine max* L. (Ouakfaoui *et al.* 2010), and *Theobroma cacao* (Florez *et al.* 2015) activates the signal transduction pathways that leads to the formation of somatic embryos (Jha and Kumar 2018). In *Arabidobsis thaliana, BBM* is a transcription factor for *LEC* gene expression in somatic embryogenesis process (Horstman *et al.* 2017). As a transcription factor, the expression of the *BBM* gene at certain phases of cotton plant somatic embryogenesis needs further confirmation.

As mentioned in the previous paragraph that BBM is the transcription factor of *LEC*, the expression of *LEC* was identified at 2 WAT of regeneration, which indicates that there was an *LEC* activity related to the somatic embryogenesis process in cotton plants at the beginning of the regeneration phase. The expression of *LEC* is not only regulated by the transcription factor but also hormonal signaling and chromatin modification (Salaün *et al.* 2021). Ledwon and Gaj (2011) confirmed that the presence of auxin in the media stimulated the expression of *LEC* during somatic embryogenesis. The *LEC* expression in *Arabidopsis thaliana* (Horstman *et al.* 2017) is a transcription factor that induces the somatic embryos formation in the seedling phase. In *Gossypium hirsutum* and *Gossypium barbadense, LEC* is expressed from the zygotic phase to the globular phase, while at the plant development stage, its expression will be limited (Wang *et al.* 2022).

In this study, the expression of *GhWOX11* and *GhWUS* was only confirmed on M3 media. Based on morphological characteristics, the callus regenerated on the M3 media showed no the direction of regeneration as expected. The expression of *GhWOX11* gene confirmed in *Gossypium hirsutum* L. was influenced by the presence of auxin as 2,4-D, by showing a high level of expression in the hypocotyl (Wei *et al.* 2022). In addition, the *GhWOX11* and *GhWUS* expressions on M3 regeneration media did not show regeneration results in the expected direction. In Xiao *et al.* (2018), *GhWUS* expression was identified in shoot and flower meristems of *Gossypium hirsutum* L.

The prescense of exogenous hormone also gave differet pattern of expression in fiber-related genes. The expression *F3H* showed a pattern that in line with the regeneration results in M1 and M2 media. In embryogenesis phase, the metabolite activities in cells tend to increase. This is because the active cells differentiate into cells in other formats. The *F3H* (*flavanone-3-hydroxylase*) is a key enzyme in the regulation of flavonoid accumulation and color formation in cotton fiber cells (Tan *et al.* 2013). In cotton, *F3H* was confirmed to be involved during the predominance of fiber formation with varied expression, depending on the cotton variety (Zheng *et al.* 2023). The *F3H* expression pattern indicates that the M1 and M2 media are the most effective media in the regeneration phase, whereas the M1 and M2 media had a higher auxin ratio, than the M3 and M4 media. This is because auxin is a key regulator that plays a role in activating the *F3H* transcript (Lewis *et al.* 2011).

The *GhMYB25-like* is an important gene in cotton that regulates the differentiation and development of trichome cells. The previous studies stated that the *GhMYB25-like* expression was limited in ovule epidermal and younger fiber cells, and unexpressed in other tissues, including leaves (Walford *et al.* 2011). However, the *GhMYB25-like* was expressed in the embryogenesis phase. This provides an information that the *GhMYB25-like* is involved in the embryogenesis phase of cotton plants, although it has no relationship to the regeneration level and the hormone combination application.

The expression of *GhHD-1(homeodomain-leucine zipper)* was reported as part of the HD family, that has a function as a downstream signal to stimulate the trichoma cell differentiation and cell growth (Walford *et al.* 2012; Shan *et al.* 2014). The *GhHD-1* has been reported to initiate fiber formation during cell differentiation, thus terminating this gene can reduce the trichome formation. Based on the results of this study, the *GhHD-1* expression at 2 WAT was found on the M3 media, then fully expressed in all treatments at 4 WAT. During the embryogenesis process, the activation of this gene provides an information that *GhHD-1* is present during the embryogenesis process. The *GhHD-1* may indicate the correlation of this gene and cotton fiber character, although requiring a further confirmation.

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