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Transcriptome analysis of defense related-gene in bananas (*Musa acuminata*)

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Abstract. *Banana is one of the prime tropical plants from Indonesia, but there is a problem of the amount decreases of production caused by disease attacks on plants from Fusarium oxysporum f. sp. Cubense. One of prevention is knowing the pattern of early expression on banana plants due to fusarium wilt disease to get the initial profile of the banana plant defense gene associated with phytohormone. The work phase starts with the primary design then with Foc conidia culture on PDA and PDB media. Plants are then infected with conidia and isolated RNA. Preparation of cDNA was done with RNA isolation result then PCR was done with primer that had been design and quantified with electrophoresis. The banana plants infected with Fusarium conidia has a different phenotypes with control plants, banana color changes become yellowish already seen since the 6th day on plants with treatment. Observation of plants up to day 40th shows the difference of outer appearance between plants in Foc infection and control plants, dryness in plants with treatment. Electrophoretic bands ERF1, ERF2, and AXR1 have down regulation. ETR1 and GA3 have over expression. The height of gene expression in both treatment and control depends on the functionality of each gene.*

Key words: Banana*, Defense Gene, Fusarium oxysporum, Musa acuminata*, *Transcriptome*

INTRODUCTION

Banana is one type of tropical plants that can grow in various regions in Indonesia but there is a problem of production in the industry caused by *Fusarium oxysporum* f.sp *Cubense* (*Foc*). These plant disease is the most dangerous disease for banana in the world because not only cause of wilting but can also cause death in plants (Visser 2010). Several hectare of banana farming in Asia, Australia, and America are destroyed due to attacks from this pathogen. Banana have different genome such as AA, AAA, AB, AAB, and ABB. Barangan (*Musa acuminata*) is triploid AAA whis is vulnerable to *Foc* (Li *et al*. 2013).

Fusarium wilt is one of the most dangerous plant diseases in the tropics. In 1940, the disease had damaged the production of Gros Michel banana exports in Central America and the Caribbean in large numbers. Not only type Gros Michel, *Fusarium oxysporum* f.sp. *cubense* (race 4) is also able to attack other types of bananas (Agrios 2005). The Symptoms in infected banana by *Foc* are characterized by brownish-colored necrosis of the vascular tissue and yellowish on the leaf (Ploetz 2006). Farmers usually using pesticide to protect their plant, but it is not effective to against the pathogen because it can adverse the environment.

Detect early symptoms on banana plants due to *Foc* attack (Fusarium wilt) is one of the way to prevent spreading of pathogen. The initial response of plants to a stress can be observed through the process of transcription of DNA into mRNA as a molecular mechanism occurring within the cell. Transcript of mRNA (transcriptomic) may be an indication of the activation or inhibition of a gene within a particular cell and tissue in response to stress. Differences in mRNA transcripts on infected plants compared to plants can not describe the condition of the plant. Utilization of this can be used as a strategy to reduce the spread of disease (Morcillo *et al*. 2006).

Plants always generate responses to defend against pathogens (Nürnberger & Lipka 2005), one of which is to regulate the signaling branches regulated by phytohormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) genes related to active plant defense (Sheard *et al*. 2010). These three hormones are produced in plants along with the infection of pathogens and synergistically work with defense-related gene expression such as *ERF1* (*Ethylene response factor 1*), *ERF2* (*Ethylene response factor 2*), *ETR1* (*Ethylene receptor 1*), *GA3* (*Gibberellin 3*), and *AXR1* (*Auxin resistence 1*). The expression of hormone-related defense genes will provide information on the response of plants to disease (Morcillo *et al*. 2006).

The aim of this research was to obtain a description of plant phenotype infected by *Foc* and to know the pattern of early expression profile of banana defense defense response gene associated with fitohormon, especially jasmonic acid hormone (JA), salicylic acid (SA), and ethylene (ET). The results of this study is useful to determine the information of early symptoms of plants after *Foc* infection.

MATERIALS AND METHODS

Study area

The research was conducted in February 2017 until June 2017 located in molecular laboratory and green house of Al-Azhar University Indonesia. Banana plants aged 30-45 days were taken from the Tissue Culture Laboratory Lebak Bulus already acclimatized previously adapted in green house.

Procedures

Primer Design

Primers used for PCR are designed on the basis of conservative areas of defense-related hormones such as AUX (auxin), GA (giberelin), JA (jasmonate) and ET (ethylene) in some plants contained in the Banana genome database CIRAD (www.banana-genome- hub.southgreen.fr) GENE BANK or NCBI database with BLAST program (www.ncbi.nlm.nih.gov) and multiple alignment on Clustal W Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Slameto & Sugiharto 2010). The RNA sequence is analyzed to find the primary candidate using the PRIMER3 program (http://primer3.ut.ee/). Primary and efficiency as well as the presence of primary dimer were analyzed using NETPRIMER (http: //www.premierbiosoft.cpm/netprimer/). Primer that has been designed then ordered and tested its activity by performing electrophoresis.

Foc Culture Preparation

The first step is to inoculate *Foc* for approximately 7 days on PDA and PD. Conidia is filtered using filter paper and then washed with distilled water. Before the density is calculated to 106 conidia /ml using a haemocytometer, conidia is first dissolved in the aquadest. The next step is weighing the *Foc* mycelium as much as 2 grams and dissolved in 200 ml aquades. After weighed, conidia then infected on the seeds of banana plants.

Foc infection to plant treatment

The 21 Banana plants planted and cleaned roots using water. A total of 18 plants were given treatment and 3 other plants were used as controls that were not given treatment. The treatment group was divided into 6 groups namely H1, H2, H3, H4, and H5, and H6. The initial treatment was performed by immersing the five groups of plants within 200 ml of Foc conidia suspension for 30 minutes, while the control plants were soaked for 30 minutes in the aquades. After soaking the plants are replanted in the soil (Li *et al*, 2013).

Observation of Banana Phenotype

After *Foc* infection on banana is observation of banana phenotype. Observation of banana phenotype done every day to see the appearance of symptoms of Fusarium wilt disease. The observation held every day for 40 days.

RNA Isolation

Total RNA isolation was taken from the leaf section using GENEzolTM. The reagent is then purified by phenol chloroform isoamite (PCI) 25: 24: 1 in order to remove secondary metabolites from the sample. A total of 50-100 mg samples were smoothed with a grinder by the aid of liquid nitrogen, then transferred to a 1.5 ml sterile tube. The next step is the addition of 1 ml of GENEzolTM Reagent, followed by centrifugation process at 5000 rpm for 10 minutes. The supernatant obtained from the centrifugation was then added 0.5-1 ml PCI, then centrifuged for 13,000 rpm within 5 minutes. The coloration of supernatant color obtained using PCI as much as 2-3 times to be clear.

The purified sample with PCI was then added 200 µl of chloroform and divortex approximately for 10 s, then centrifuged at 12,000 rpm for 10 min. The next step is to precipitate the supernatant by adding 1 volumes of cold isopropanol and incubated for 10 minutes at room temperature. The result is then centrifuged at 12,000 rpm for 5 minutes. The supernatant phase is then discarded and the pellet obtained is the total RNA isolated. The pellet is then washed with 1 ml of 70% alcohol and centrifuged for 5 minutes at a speed of 12,000 rpm. The centrifugation result was then dried at room temperature for 5-10 minutes and resuspended using 50 µl ddH2O.

cDNA Synthesis

Stages performed after total RNA resuspension with 50 µl ddH2O were quantified using MaestroNano Spectrophotometer (Maestrogen Inc, http://www.maestrogen.com). After we get the good quality of RNA it is taken as much as 5 µg for cDNA synthesis. Synthesis was performed using 1 µl primary random hexamer, 1 µl dNTPs, 5 µl buffer reverse transcriptase and added ddH2O to reach 20 µl volume then incubated at 37C for 60 min.

Semiquantitative PCR Amplification

Semi-Quantitative Amplification PCR cDNA was taken as much as 2 µl to be used as PCR reaction template with Biometra PCR thermocycler. The housekeeping genes used for amplification are the ACTIN gene. The PCR composition for amplification was 2 µl total cDNA, 12.5 µl PCR mix, 1 µl primers (forward and reverse) and H2O with 20 µl reaction volume. The PCR process was pre-PRCR at 95 ° C. for 5 minutes, denaturation at 94 ° C. for 30 seconds, the primary

attachment at 57 ° C. for 30 seconds, and elongation at 72 ° C. for 1.5 minutes, with 35 cycles, and post PCR at 72 ° C, 5 min, followed by 15 ° C, 10 min later PCR results in electrophoresis to see its gene band (Suharsono et al., 2008).

Data analysis

Gene expression patterns were analyzed quantitatively using the ImageJ program (Abramoff *et al.* 2004). First the intensity analysis of Ladder DNA band 1kb from Geneaid to be a reference concentration on the experimental results. Once known, each band of electrophoresis in crop then processed with ImageJ (https://imagej.nih.gov/ij/) to know the intensity to know the concentration on each band. Concentration results then analyzed with Microsoft Excell and created in the form of concentration pattern graphs.

RESULTS AND DISCUSSION

Results and Discussion should be written as a series of connecting sentences, however, for manuscript with long discussion should be divided into subtitles. Results should be clear and concise.

Plant phenotype infected by *Foc*

Observations on banana plants showed the leaves on 6th day after infection with *Foc* change discoloration on the lower leaves (older leaves) or commonly referred to as symptoms of chlorosis. According to Ploetz (2006), bananas that have been infected by *Foc* will experience symptoms of leaf chlorosis to yellowish on the lowest leaves. *Foc* pathogens will spread through the roots and move throughout the plant to block the vascular system, causing the yellowish color of the lower leaves followed by other young leaves to dry and ending at the death of the plant. Based on observations on plants, symptoms of post-infectious *Foc* disease look very fast. On the 6th day (Figure 1) chlorosis has occurred surrounding the lower leaf portion to a yellowish color. Plants that have been attacked will change slowly into bright yellow before it died, the spread of this disease usually spread quickly. The extermination of Foc races 4 that has attacked banana plants and plantations is not possible because *Foc* that attack plantations will rapidly destroy entire plantations (Cooperative Research Center Plant Biosecurity 2009).

Fusarium oxysporum f.sp *cubense* (*Foc*) race 4 can attack any type of banana genome that is attacked by other Fusarium races, while other Foc races can only attack multiple genomes. Foc race 1 can attack bananas with the genomes AAA, AAAA, and AAB. Foc ras 2 can only attack AAAA and ABB bananas while *Foc* race 3 only attacks ornamental plants (Nasir *et al*. 2003). Therefore an effective way to be done by farmers is by eradication of plantations because there is no banana genome that is resistant to *Foc* attacks.

Observation of plant leaves after *Foc* infection experienced a yellowish color change on the 7th day. The yellow color changes appear to be spreading as in Figure 2, day 17 shows the discoloration of the leaves from the yellowish to dry brown. *Foc* that infect plants will block the vascular system and inhibit the translocation of water and nutrients from the roots to all parts of the plant. This causes the plants to dry and die (Department of Agriculture and Fisheries Biosecurity Queesland 2015).

Figure 1. Phenotype of plant on the 6th day (A) Control banana plant (B) Banana plant after infection *Foc* (C) Banana leaf control (D) Banana leaf post infection *Foc* (E) Banana leaf post infection *Foc* Yellow color on leaves show symptoms chlorosis due to post-infection damage.

In plants infected by *Foc*, the process of yellowing does not occur simultaneously on all leaves but occurs gradually starting from the oldest leaves. The oldest leaves are at the bottom of chlorosis first. The yellow color widened in the 7th day on the first leaf and was visible widened on the 9th day on the second leaf. The change of leaf becomes dry faster occurs on the first leaf on the 17th day, while on the second leaf has not changed to complete dry on the 17th day (Figure 2). This can happen because Foc attacks the plant from the roots, so the blocking system starts from the leaf closest to the root (Ploetz 2006). In contrast to plants infected by *Foc*, the first and second plant leaves on the control plants showed no symptoms of discoloration to a bright yellow until the 17th day.

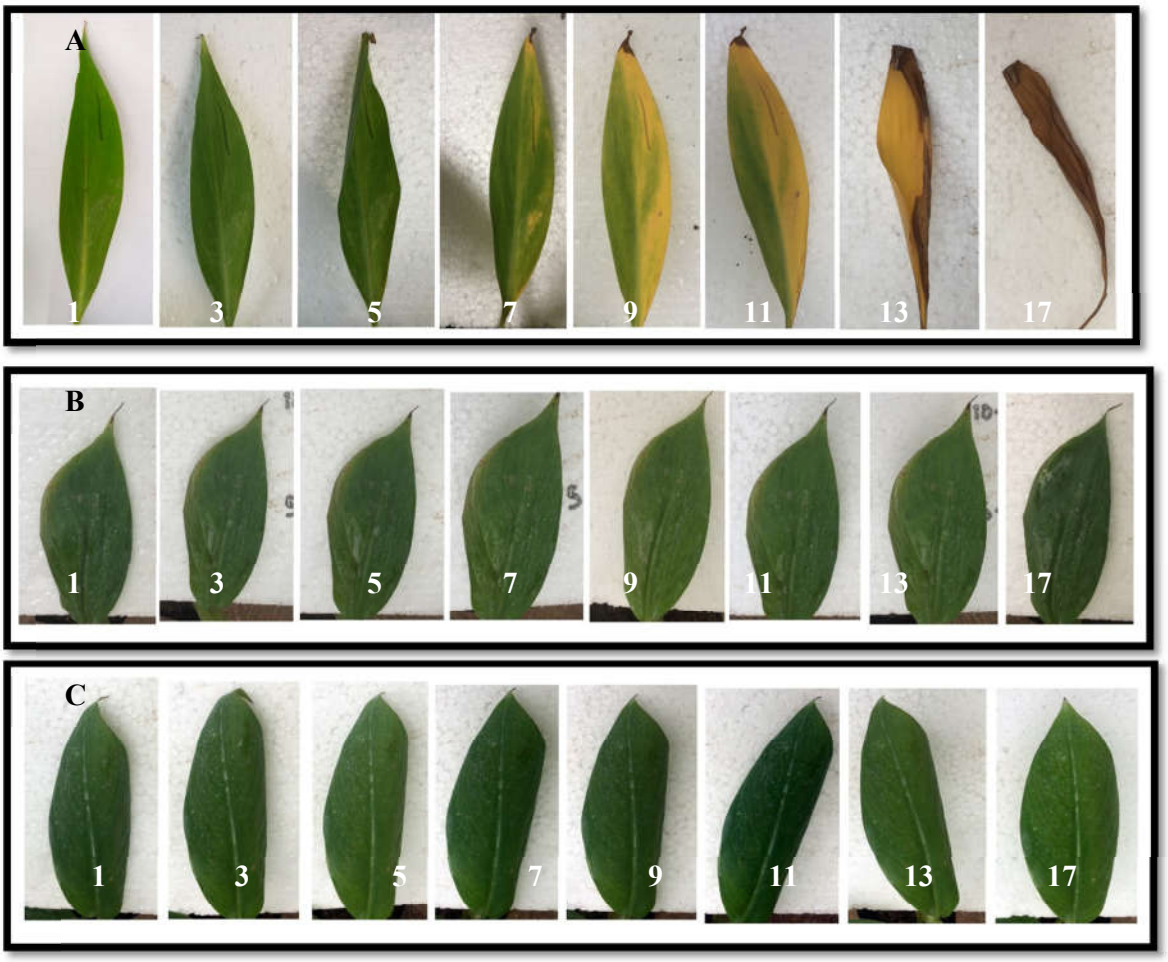

Figure 2. Leaves of banana plants after *Foc* infection and control on day 1,3,5,7,9,11,13 and 17. (A) The oldest leaf which is the lowest leaf on plant post infection Foc (B) 2nd leaf of the lowest part of the plant post infection *Foc* (C) The oldest leaves in the control plant (D) The second leaf from the bottom. The green leaf changes yellow and dries to brown, indicating post-infection damage.

Figure 3. Control plants and treatment plants (post *Foc* infection) on day 20 and day 40. (A) Control plants on day 20 (B) Control plants on day 40 (C) Plant by treatment on the 20th day (D) Plant by treatment on day 40.

Phenotypic observation of banana plants was done until day 40. In addition to the leaves, the differences were clearly seen in the whole plant between the treatment plants and the control plants. On the 20th day the treatment plant showed several leaves that had withered and the split rod, while the control plant had no leaves withered and the split rod (Fig. 2). This is in accordance with a report from Monila *et al*. (2011) that the emergence of split rods is one of the plants affected by *Foc*. Plants that have been attacked by *Foc* will rapidly spread the disease throughout the plant, after experiencing the yellow color changes in all the leaves, a characteristic feature of the plant infested by *Foc* is splitting the stems then there will be drying and death on the plant as shown in plant post-infection day 40. All the colors on the leaves turn brown and dry (Figure 3D).

Semiquantitative Analysis

Semi-quantitative expression analysis of PCR with several genes such as *ERF1*, *ERF2*, *ETR1*, *AXR1*, and GA3 have different results. Differences in gene expression are represented by changes in band thickness on different days (Figure 4). *ERF1* in treatment increased expression on day 3 then again decreased on day 4 and increased on day 6. In contrast to treatment, expression on *ERF1* controls showed higher expression from day one, then increased on day 4 and decreased on day 5. *ERF2* in treatment had increased expression on day 4 and decreased expression on day 5, while expression on control increased on day 4 and decrease on day 5 as happened in *ERF2* control.

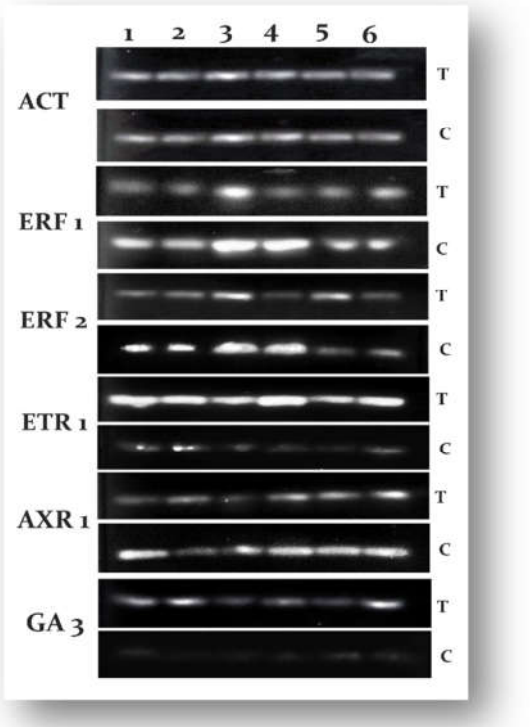

Figure 4. Semiquantitative Expression of ACT, ERF1, ERF2, ETR1, AXR1, GA3 on banana post infection *Fusarium oxysporum* f.sp *cubense*. (T) treatment (C) control (1) day 1 post infection (2) second day post infection (3) day 3 post infection (4) post-infection day 4 (5) day 5 post infection (6) 6th day post infection.

In contrast to other genes, *ETR1* experienced high expression since the first day after Foc conidia infection slightly experienced a decrease on day 5 and again increased on day 6. Controls on *ETR1* have a low expression since day 1 then increase on day 2 and decrease again on day 3. The expression of the *AXR1* gene on the treatment increased on the 4th day until the 6th day, whereas the controls showed a high expression from day one and decreased on day 2. Improved expression of *AXR1* control is again visible on Day 3 to 6th. The gene expression that occurred in *GA3* decreased on day 3 and increased again on day 6, while the controls have a constant and low expression from day 1 to day 6. The expression pattern of the ribbon is converted in graphical form in Figure 5.

Ethylene is a simple gas that is heavily involved in plant physiology. Ethylene is the most important modulator in defense against plant diseases. Generally, signal communication on ethylene has a positive role in resistance against pathogens such as Fusarium oxysporum (Van loon *et al.* 2006). Increased expression of *ERF1* in plants is a transcription that activates ethylene responsive genes that can increase resistance to pathogens. Based on research conducted by Li et al. (2013) in Arabidopsis, ERF gene was found with high expression after infection by Fusarium oxysporum f.sp cubense (*Foc*) after day 2. In accordance with these studies, increased expression of *ERF1* and *ERF2* in banana plants can occur due to the defense performed by post-infectious ethylene and looks high on day-to-3. The existence of the defense then allows the decrease of expression on the day after that is the 4th day.

Genes *ETR1* cooperates in signaling with other genes and hormones in response to stress, one of which is PR (Pathogenesis related). High ethylene signaling can increase resistance to pathogens. The expression of ETR in tomato plants infected by Ralstonia solanacearum was elevated followed by rapidly increasing expression of the PR gene, but a higher and faster expression was shown in tomato plants infected by Xanthomonas campestris (Zhang et al., 2004). Increased expression of ETR also occurs in tobacco plants affected by *Peronospora hyoscyami* f. sp. *tabacine*, but did not experience a sensitive response to tobacco plants attacked by the Tobacco mosaic virus (Geraats *et al,* 2003). This suggests that the same genes in the same species may produce different responses, depending on the type of pathogen.

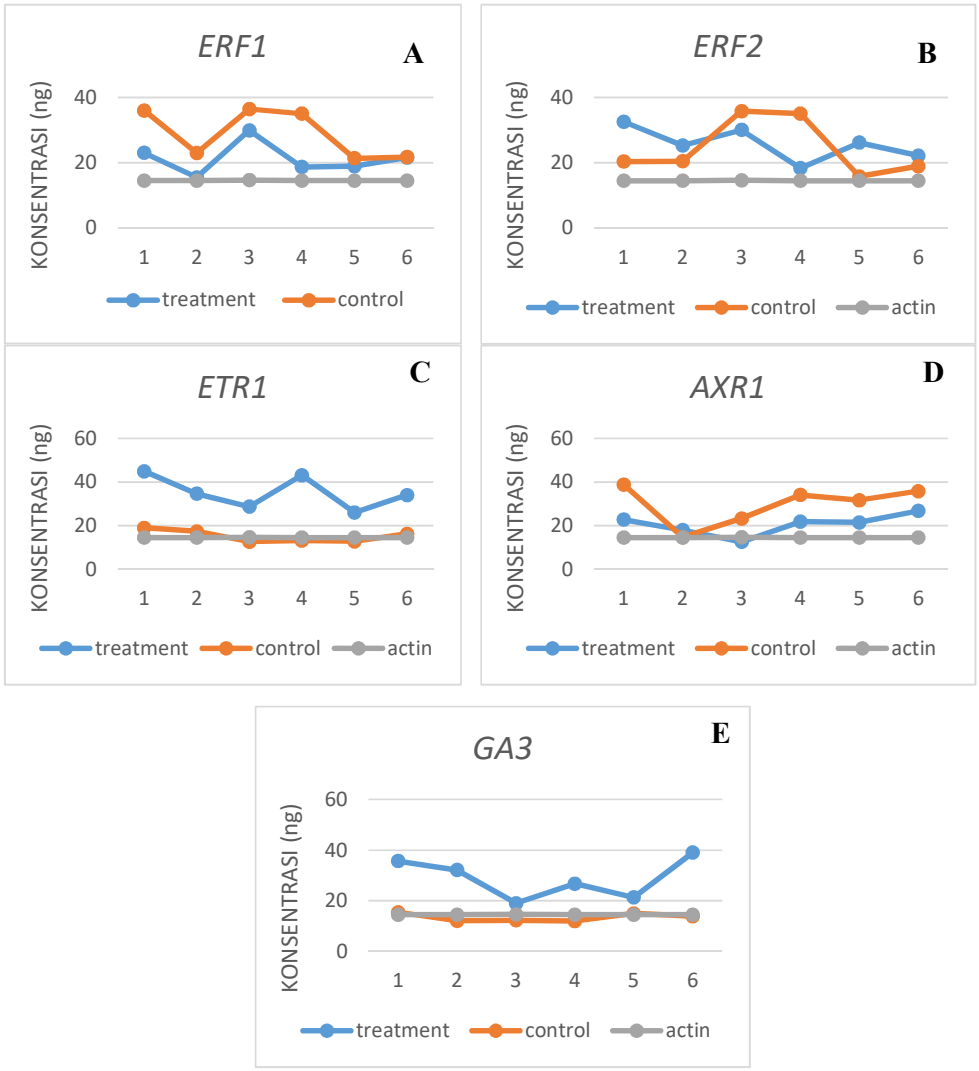

Figure 5. Graph of expression pattern on *ACT, ERF1, ERF2, ETR1, AXR1, GA3* genes. (A) Expression pattern on actin, control, and treatment on *ERF1* (B) Expression pattern on, control, and treatment on *ERF2* (C) Expression pattern on actin, control and treatment on *ETR1* (D) Pattern of expression on actin, control, and treatment on *AXR1* (E) The expression pattern on actin, control, and treatment on *GA3*. Data expression pattern is taken from the result of ribbon image analysis with ImageJ.

Discussion

Phenotypic observations on banana plants showed symptoms of chlorosis on the 6th day post Foc infection in the form of discoloration of the leaves to yellow and change color to brown on the 17th day on the lowest leaf and followed by chlorosis in younger leaves. Observation of plants up to day 40 shows the difference of outer appearance between plants in Foc infection and control plants, control plants are still green until day 40 while plants that have been infected are brown and dry. The expression of semi-quantitative real time PCR with several genes such as ERF1, ERF2, ETR1, AXR1, and GA3 have different results. ERF1 experienced an increase in expression on day 3, ERF2 had an increase in expression on day 4, ETR1, decreased on day 5 AXR1 experienced a 4th and 6th day, and GA3 decreased on day-to- 3. The height of gene expression in both treatment and control depends on the functionality of each gene.

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