The Expression of Glucanase Encoding Gene *(CaβGlu)* in Chili (*Capsicum annuum* L.) As a Response to *Fusarium oxysporum* Infection.

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ABSTRACT

Indonesia is a tropical country with highest level of biodiversity, especially in the agricultural sector. Chili (*Capsicum annumm*. L) is a very well-known and widely used agricultural product in the world, which makes chili becomes one of the most considerable national product. The chili production is oftentimes very susceptible to some diseases caused by virus, fungi, or bacteria. One of the most common diseases in chili cultivation is Fusarium wilt, which is caused by *Fusarium oxysporum*. This disease can cause a major loss and up to 50% crop failure. Many procedures have been done to find the best cultivar with a resistance trait to *Fusarium oxysporum,* including by observing and testing the chili's genetic resistance. One of the resistance genes in chili is β -1, 3-glucanase-encoding gene, which produces an enzyme to hydrolize the cell wall of pathogenic fungi. This research aimed to determine the expression of the glucanase-encoding gene (*CaβGlu*) in chili as a response to *Fusarium oxysporum* infection. The methods including chili cultivation, *F. oxysporum* inoculation, isolation of chili leaves RNA, glucanase-encoding gene expression analysis using qRT-PCR, and data analysis. The result of *CaβGlu* gene expression is higher than the control in the first 6 hours after inoculation, and decreasing in the 48th and 96th hours. The conclusion was the infection of *Fusarium oxysporum* is activating the expression of *CaβGlu* gene which was expressed best in the first 6 hours after inoculation.

Keywords: *Capsicum annuum L,* , Ekspresi gen, *F. oxysporum*, Gen penyandi glukanase *(CaβGlu),* qRT-PCR

I. INTRODUCTION

Chili (*Capsicum annuum* L.) is a very well-known and widely used agricultural product in the world, which makes chili becomes one of the most considerable national product. *Fusarium oxysporum* is one of the factors causing the , diseases. Rostini (2011); Mahartha et al., (2013), states that *Fusarium oxysporum* can cause losses and crop failure up to 50%. Various methods are used to find chili cultivars that resistant of Fusarium oxysporum, one of study the genetic resistance of chili by testing the resistance genes that are owned by chili plants, so that it can be known how the expression of these resistance genes in response to disease pathogenic genes that enter in the body of a plant. One of the resistance genes in chili is β -1, 3- glucanase- encoding gene, can hydrolyze the cell walls of pathogenic fungi that infect plants. β -1, 3- glucanase- encoding gene is one of the plants defense genes against fungi can inhibit of pathogenic fungi (Shaikh, 2005; Winangsih, 2014). β-1,3-glucanase-encoding gene is expressed as an important component in the defense

mechanism of fungal pathogenic. This research aimed to determine the expression of the glucanase-encoding gene (*CaβGlu*) in chili as a response to *Fusarium oxysporum* infection.

MATERIAL AND METHODS

Fungal and Plant Material

Pathogenic *Fusarium oxysporum* was isolated from rejuvenation of Biotechnology laboratory collection. The fungi were grown in Potato Dextrose Agar and Broth for the cultivation before inoculated to plants. This research used local Indonesian red-chili cultivars is Lembang -1 by by Indonesian Vegetable Research Centre. RNA isolation use Plant RNA Mini Kit (Geneaid). qRT-PCR use Bioline Bio – 72001 sensiFAST SYBR No-Rox One Step Kit, 100 rnxs – 1 Qty, reverse and forward primer of glucanase gene and 18S rRNA as normalisator.

METHODS

Inoculation of *Fusarium oxysporum*

Inoculation of *Fusarium oxysporum* on chili plant after 30 days growth. Treatment plants are soaked by *Fusarium oxysporum* suspension and the control plants is not. *Fusarium oxysporum* was grown in Potato Dextrose Broth (PDB) for seven days incubated up to $10^5 – 10^6$ conidia/mL. Spore density was calculated with a hemocytometer. The conidia were inoculated on 30-day-old chili plants by the root dip method.

RNA Isolation

RNA isolation was carried out on chili plants that had been inoculated with *Fusarium oxysporum* and compared with controls using leaves. Isolation was carried out on day-0, day-2 and day-4 postinoculation. The processed RNA isolation use *Plant RNA Mini Kit Geneaid.* Leaf samples were weighed as much as 0.1 gram and then cooled at -20^o C before RNA isolation. The sample is ground into a fine powder and then transferred to a 1.5 ml microcentrifuge tube. Step of cell lysis begins with added 500 µl of RB Buffer in microtube, mix by vortex. Incubate at 60ºC for 5 minutes. Place a Filter Column in a 2 ml Collection Tube and then transfer the sample mixture to the Filter Column. Centrifuge for 1 minute at 4000 rpm then discards the Filter Column with carefully transfer the clarified filtrate to a new 1.5 ml microcentrifuge tube. The sample was added ½ volume of absolute ethanol to the clarified filtrate then shake vigorously. Place a RB Column in a 2 ml Collection Tube then transfer the mixture to the RB Column. Centrifuge at 12000 rpm for 1 minute. Discard the flow-through then place the RB Column back in the 2 ml Collection Tube. Add 400 µl of W1 Buffer into the center of the RB Column. Centrifuge at 14-16,000 x g for 30 seconds. Discard the flow-through then place the RB Column back in the 2 ml Collection Tube. Add 600 µl of Wash Buffer (make sure ethanol was added) to the center of the RB Column. Centrifuge at 12000 rpm for 30 seconds. Discard the flow-through then place the RB Column back in the 2 ml Collection Tube. Add 600 µl of Wash Buffer (make sure ethanol was added) to the center of the RB Column. Centrifuge at 12000 rpm for 1 minute. Discard the flow-through then place the RB Column back in the 2 ml Collection Tube. Centrifuge at 12000 rpm for 3 minutes to dry the column matrix. The RNA elution step is carried out by placing a dry RB column in a clean 1.5 ml microcentrifuge tube. A total of 50 µl of RNase free water was added to the center of the column matrix. For 2-3 minutes the column matrix is left to ensure the RNase free water is completely absorbed. With a speed of 12000 rpm, the sample is centrifuged for 1 minute to elute purified RNA. The concentration and total purity of RNA were measured spectrophotometrically using NanoDrop instruments and purity was assessed using the A260 / 280 and A260 / 230 ratios determined by NanoDrop (Zhang et.al., 2013).

Total RNA Dilution

The isolation RNA was further measured in concentration and purity using NanoDrop at 260/280 wavelengths. After knowing the concentration and purity then the total RNA was diluted before PCR was done to make the concentration of RNA samples relatively the same. RNA results of the isolated samples were measured at wavelengths of 260 nm to determine the total RNA concentrations of each sample. These total RNA levels are used to equalize the amount of RNA to be used in RT-PCR approximately 50 µgper sample by adjusting the volume and dilution of the total RNA used in each RT-PCR mixture.

Proses *Quantitative Reverse Transcriptase* PCR

The qRT-PCR reaction is carried out through a single reaction stage using Bioline Bio-72001 sensiFAST SYBR No-Rox One-Step Kit, 100 rnxs-1 Qty. Primer pairs used in the selection of the qRT-PCR reaction are glucanase genes for target genes and 18S RNA gene primers for normalizer genes or housekeeping primers (Zhang et al., 2013). The base sequence in the 18S rRNA primer is as follows, in 18S RNA F is 5'-GCTTAATTTGACTCAACACGGGA -3 ', and the primary sequence in 18S RNA R is 5' - AGCTATCAATCTGTCAATCCTGTC-3 '. Amplification of glucanase genes was carried out with forwarding primers with the following base sequence glu - F was 5' TAAAAGGGGAAGTCCAAGAAGG-3' and the base sequence used for reverse primers was glu - R 5'TCAGCAAAAATGTCCAAAATC-3' with a size of 180 bp (Zhang et.al, 2013). The qRT-PCR mixture was made with Bioline Bio - 72001 sensiFAST SYBR No - Rox One - Step Kit, 100 rnxs - 1 Qty total volume of 20 µl / reaction. The composition of the mixture is 10 µl 2x SensiFAST SYBR No-ROX One-Step Mix, 0.8 µl 10 µM forward primer (400nM), 0.8 µl 10 µM reverse primer (400 nM), 0.2 µl reverse transcriptase, 0.4 µl RiboSafe RNAse inhibitors, 16 µl µl H2O and 4 µl template.

The temperature is set at 45ºC for 10 minutes for the reverse transcription process (cDNA synthesis), followed for 95ºC activation for 2-5 minutes for RT activation, 95ºC for 5 seconds for denaturation, 55ºC 10 seconds for annealing (primary attachment), and the cycle is repeated 40x, and a final elongation of 72ºC for 5 minutes (Zhang, et.al., 2013). Quantification of glucanase gene expression was carried out simultaneously on each sample of treated plants and compared with the expression of control plants by the delta-delta CT method (Livak & Schmittgen, 2001). The analysis was performed with the GeneQ Software Rotor software version 2.1.1. The qRT-PCR analysis method refers to (Zhang, et.al., 2013) which is performed using a specific combination of gene-specific primers for each cDNA sample. qRT-PCR was carried out with a PCR Detection System using Bioline Bio - 72001 sensiFAST SYBR No - Rox One - Step Kit, 100 rnxs - 1 Qty. A comparison of gene expression is calculated by the delta - delt Ct method.

RESULTS

Tabel 1. The results of RNA Purification on Chili Leaves

Sample	Concentrasi (µg/mL)	A 260/280
K H0	216.6	2.07
P H0	146.3	1.97
K H2	123.2	2.14
P H2	200.7	2.15
K H4	428.0	2.12
P H4	480.9	2.09

** K: Control Plant, P: treatment plant, H0: day-0, H2: day-2, H4: day-4*

Table 2. The Results of RNA Purification on Chili Leaves after Delution

Sampel	Concentration(µg/mL)	A 260/280
K H0	41.7	2.07
P H0	43.6	1.97
K H2	48.2	2.14
P H2	59.3	2.15
K H4	48.3	2.12
P H4	44.7	2.09

** K: Control Plant, P: treatment plant, H0: day-0, H2: day-2, H4: day-4*

Table .3. qRT-PCR analysis of the glucanase-encoding gene (CaβGlu) of the Lembang-1 cultivar

Replicate Name	GOI CT	Norm. CT	Relative Conc.	Rel Min	Rel Max	Calibrator
DdCt H0						
P	21.86	13.64	19.17	19.17	19.17	
K	25.76	13.28	1	1	1	Yes
DdCt H2						
P	22.10	18.44	2.25	2.25	2.25	
K	22.14	19.64	1	1	1	Yes
DdCt H4						
P	20.03	19.99	1.45	1.45	1.42	
K	20.24	19.66	1	1	1	Yes

**DdCt = Deltadelta cycle threshold, GOI = Gene of interest, NORM = Gene Normalisator, H0 = day-0, H2 = day- 2, H4 = day- 4.*

DISCUSSION

RNA isolation was carried out in 3 times, ie on plant samples day 0 (hour 6), day 2 (hour 48) and day 4 (hour 96). The results of RNA isolation are stored in the freezer to keep it from being denatured, the RNA purity test uses a quantitative nanodrop spectrophotometric test with an absorbance ratio at 260/280.The results of RNA of chili leaves in table 1. The ideal RNA purity is obtained from the RNA isolation process that is equal to 95-100% with an absorbance ratio of 2.00 ± 0.05, RNA with A260 / A280 ratio value equals 2 has a purity level of 100%. Based on nanodrop quantitative test results, the concentration and purity of RNA of chili leaves were tested using a spectrophotometer. The value of absorbance 1 at a wavelength of 260 nm is equivalent to 40 µg RNA / mL.

The RNA concentrations obtained were not uniform, ranging from 80 - 650 µg / mL with purity obtained from 1.8 to 2.1 (Table 1),. The purity value of RNA (A260 / 280) is influenced by pH. Measuring the purity of RNA with the nuclease-free water solvent with an irregular pH will often experience changes ranging from 1.97-2.19. Pure RNA has A260 / 280 1.8-2.1 values when dissolved in Tris-Cl 10 mM pH 7.5 (Qiagen 2000). All samples

have good purity and do not undergo DNA or protein contamination. This is consistent with the literature, where the ideal purity of RNA with a value of 95-100% if an absorbance value of 2.00 ± 0.05 is obtained. The concentration of RNA obtained from each sample has a significant difference of between 80 - 650 µg / ml, to obtain the cDNA value in real-time PCR, the results of RNA isolation must be equalized. The equalization of concentration values is done by dilution using the addition of nuclease-free water (RNAse free water).
The use of RNA isolation results for quantitative real-time PCR must be prioritized to concentrate, this aims to avoid bias values on the results and if there is a very significant difference in concentration it will affect the intensity of the amplified value formed, therefore the RNA concentration of all samples is equalized through dilution to 50 µg / ml on each sample with RNAse free water added. Based on the results of dilution obtained uniform RNA concentrations approaching ± 50 µg / ml (Table 2). The qRT-PCR analysis is based on the Ct (cycle threshold) value determined by adjusting the threshold line to avoid the bias value and the multi Ct value. The type of software used to analyze the results of Ct values is the Rotor GeneQ Software version 2.1.1 which shows the level of expression in the Lembang Cultivar-1 chili plant (Table 3).
The level of expression of the treated plants was calculated relatively compared to the control plants which functioned as calibrators in the qRT-PCR analysis. The control plant expression level value is set at 1 in the calculation of the relative expression level (relative concentration in the qRT-PCR analysis table). Based on Table 3. Then we can graph the expression of glucanase (CaβGlu) gene encoding chili Lembang-1 cultivars (Figure 1).
The results of the analysis of the relative value of the glucanase encoding gene (CaβGlu) are 1.45 - 19.17 with GOI values Ct 20.03 - 25.76 and Norm Ct 13.28 - 19.99. Madigan, *et al* (2009) stated that gene expression was analyzed by comparing the Ct value of the target gene (GOI) with the housekeeping gene (Norm), which was determined by a threshold to determine the amplicon value so that quantitative data based on the number of copies was obtained. number) genes from calculations using a standard curve.

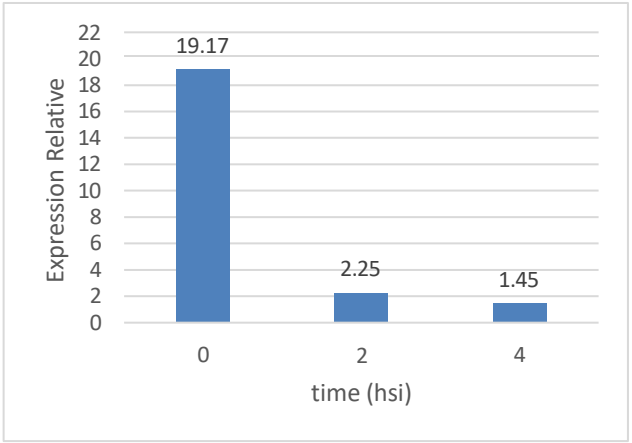

Figure 1. Expression of Glucanase –encoding gene after infection with *Fusarium oxysporum*

Expression values indicate changes in the value of the results of plant expression in responding to the *Fusarium oxysporum* fungus which continues to decline. Based on Zhang et al. (2013) there were different regulation of glucanase-coding genes in root organs and leaves, the expression of glucanase-coding genes (CaβGlu) in leaves was higher than in

plant roots, this was evidenced by the high expression value in leaf organs compared to root organs in infected chili plants pathogen. Plants respond to the initial expression of glucanase to counteract the entry of pathogenic fungi, high expression values on day 0 (hour 6) indicate that glucanase in plants responds quickly and can regulate glucanase-coding genes to the highest level. The gene encoding for glucanase is expressed constitutively by chili plants, according to Graham & Sticklen (1993) the gene for glucose encoding in plants is constitutive, but in some other plants, it is inducible. Glucose on the fungal cell wall will be hydrolyzed by glucanase produced by chili plants. Yanlin (2005) states that the beta-1, 3-glucanase gene is associated with pathogenesis involved in the initial defense system against pathogen infection.

The results of gene expression on day 2 (48 hours) showed a decreased expression, indicating that the regulation and activity of glucanase-coding genes on day 2 (48 hours) had begun to weaken or low. Day 4 (hour 96) after infection with glucanase-coding gene expression dropped to 1.45 because the chili plants included in cultivars are prone to death. Glucanase-coding genes have decreased expression where the level of expression is constantly decreasing at all times from infection day 2 (hour 48) up to day-4 (hour 96) and is expected to continue to decline until the plant dies. Plants show an initial defense response to the entry of pathogenic fungi by hydrolyzing the constituent compounds of the fungal cell wall, namely chitin and glucan (Tian *et al*., 2006). Based on research by Tian *et al.,* (2006) the expression of glucanase-coding genes on Jujube fruit showed responsive responses when infected with pathogens, and glucanase genes play a role in the process of hydrolysis of pathogen cell walls in the form of glucan carbohydrates in which the level of gene activation and accumulation of enzymes in stimulated plants thus experiencing a significant reduction in the incidence of disease-causing death in plants.

The expression test results according to the study of Zhang, *et.al* (2013) expression of glucanase-coding genes (CaβGlu) showed higher levels of expression compared to control plants. Expression of glucanase (CaβGlu) gene is triggered very quickly in susceptible plants infected with pathogenic fungi so that gene expression shows a large yield value on day 0 (hour 6) hpi.

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