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# The expression of glucanase encoding gene *(CaβGlu)*in chili (*Capsicum annuum* L.) as a response to *Fusarium oxysporum* infection

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A B S T R A C T

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 used 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.*, Gene expression*, F. oxysporum,* Glucanase-encoding gene *CaβGlu* , qRT-PCR.

## I. INTRODUCTION

Chili (*Capsicum annuum* L.) is a very well-known and widely used agricultural product in the world, 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 used to find chili cultivars that resistant of *Fusarium oxysporum*, study genetic about 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 pathogenic fungi (Shaikh, 2005; Winangsih, 2014). β-1,3-glucanase-encoding gene is expressed as an important component in the defense mechanism of fungi. This research aimed to determine the expression of the glucanase-encoding gene (*CaβGlu*) in chili as a response to *Fusarium oxysporum* infection.

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## II. 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 Lembang -1, a cultivars from Indonesian Vegetable Research Centre. RNA isolation used Plant RNA Mini Kit (Geneaid). qRT-PCR used 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*

Inoculated of *Fusarium oxysporum* on chili plant after 30 days growth. Treatment plants were 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.

### 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 used to equalize the amount of RNA to be used in RT-PCR approximately 50 µg per 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 Rotor GeneQ software version 2.1.1.

## III. RESULTS AND DISCUSSION

**Tabel 1.** The results of RNA Purification on Chili Leaves

| Sample (hpi) | Concentrasi (µg/mL) | A 260/280 |
|---|---|---|
| K 6 | 216.6 | 2.07 |
| P 6 | 146.3 | 1.97 |
| K 48 | 123.2 | 2.14 |
| P 48 | 200.7 | 2.15 |
| K 96 | 428.0 | 2.12 |
| P 96 | 480.9 | 2.09 |

** K: Control plant, P: treatment plant*

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

| Sampel | Concentration(µg/mL) | A 260/280 |
|---|---|---|
| K 6 | 41.7 | 2.07 |
| P 6 | 43.6 | 1.97 |
| K 48 | 48.2 | 2.14 |
| P 48 | 59.3 | 2.15 |
| K 96 | 48.3 | 2.12 |
| P 96 | 44.7 | 2.09 |

** K: Control Plant, P: treatment plant*

**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 6 hpi | | | | | | |
| P | 21.86 | 13.64 | 19.17 | 19.17 | 19.17 | |
| K | 25.76 | 13.28 | 1 | 1 | 1 | Yes |
| DdCt 48 hpi | | | | | | |
| P | 22.10 | 18.44 | 2.25 | 2.25 | 2.25 | |
| K | 22.14 | 19.64 | 1 | 1 | 1 | Yes |
| DdCt 96 hpi | | | | | | |
| 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, hpi (hours post inoculation).*

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) (Bio-rad, 2016). 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 was 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*) was 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.

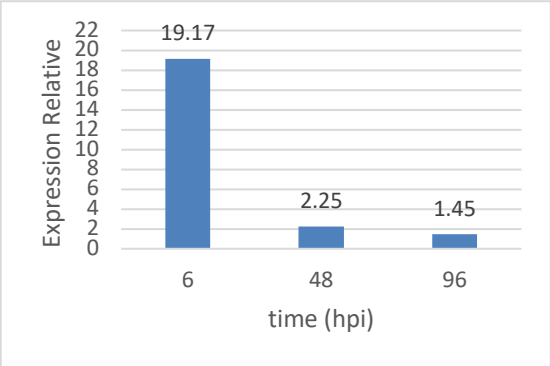


**Figure 1**. Expression of Glucanase –encoding gene after infection with *Fusarium oxysporum*

Expression values indicated 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 6 hpi, indicated that glucanase in plants responds quickly and can regulate glucanase-coding genes to the highest level. 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 48 hpi, showed a decreased expression, indicating that the regulation and activity of glucanase-coding genes on 48 hpi, had begun to weaken or low.The results in 96 hpi, 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 48 hpi up to 96 hpi 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 6 hpi.

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