The expression of chili defense gene due to oviposition of fruit fly (Bactrocera dorsalis)

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Abstract. Ovipositions of fruit flies typically leave some wounds on the chili fruits. These punctures might allow some pathogenic microorganisms to get into the fruits and eventually cause the secondary infections. Taking this into account, this study aimed at determining the expression of chili defense gene (CaRGA2) due to pathogenic fungus infection which occurred following the fruit fly oviposition activities. The research was carried out from April to October 2018 in two research facilities located in West Java, Indonesia, i.e. the Molecular Laboratory of IVEGRI (West Bandung) and the Molecular Biology Laboratory of the Biotechnology and Bioindustry Research Center (Bogor). Chili fruits of three chili varieties with different levels of resistance against fruit fly infestations (i.e. susceptible, moderately resistant, and resistant) were used in this study. Moreover, the RNA of each of the varieties was isolated before and after the fruit fly oviposition. The chili defense gene of CaRGA2 was tested in this study whereas the housekeeping gene used was Actin. The gene was amplified by using the RT-PCR method and afterwards, the data from the amplification were analyzed by employing the Livak method. The results showed that chili defense gene expression increased significantly in the susceptible chili variety, but did otherwise in both the moderately resistant and resistant varieties. Eventually, this result supported the previous studies that revealed the increased fungal pathogen infections in host plants due to the injuries caused by fruit fly oviposition.

1. Introduction

There are many challenges for chili cultivation in Indonesia, including pests attack. There would be at least 14 organisms that could potentially become chili pests [1]. One of such organisms is the fruit flies from the genus Bactrocera, which includes a large number of species. Several research have reported so far that the fruit fly species particularly associated with chili fruits were Bactrocera dorsalis [2-4]. This species oviposites their eggs, which will grow and develop into larvae, inside chili fruits. Moreover, the larvae exploit chili fruits as a source of nutrition until they are ready to become pupae. These eggs and larvae are parts of fruit flies life’s cycle inside the chili fruits, in which the cycle itself takes place in approximately 15 days [5].

The oviposition of fruit flies is an unavoidable condition for chili plants. However, this process puts the chili fruits under a biotically stressed condition. In response to stress condition, plants develop defense mechanisms which could be passive and active response [6]. The passive response is carried...
out by releasing repellent compounds or releasing volatile attractants to attract pest natural enemies. On the other hand, the active response is carried out by increasing the bioactive content of plants to reduce the survival of pests. Defense mechanism against fruit fly infestations in olive, passively by releasing volatiles compounds and actively through transcription of genes related to plant defense has been reported by [7].

Nevertheless, to date, the active response of chili fruits during the oviposition of fruit flies has not been widely reported. Previous research have mentioned about several genes related to the chili defense system against pathogens, one of which is called CaRGA2 [8]. CaRGA2 is a chili defense gene that has been isolated and characterized from CM334 chili accession, which is resistant to Phytophthora capsici [9]. When infested with the eggs of fruit flies, the fruits will decompose more rapidly due to the secondary infection by microorganisms including pathogenic fungi [10]. In the present study, we assumed that the resistance of chilies to fruit fly infestations might involve the chili defense gene against pathogenic fungi.

Thus, the aim of this research was to investigate the active response of chilies to fruit fly infestations by studying the expression of chili defense genes when infested by the eggs of fruit flies (after oviposition). The hypothesis proposed would be that the oviposition of fruit flies in chilies could activate the chili defense genes. This hypothesis was formulated based on the results of previous studies which reported that fruit fly ovipositor punctures wounded the fruits of host plants [11-13]. Moreover, the wounds could be an entry point for microorganisms, hence increasing the process of fruit rot [10]. The presence of pathogenic microorganisms in chilies would also activate the chili defense gene [9, 14]. Eventually, the results of this study were expected to support the chili breeding program by providing alternative selection methods through the defense gene expression approach.

2. Research Materials and Methods

2.1. Study site and period
The research was conducted from April to October 2018 at the research field facilities, the Plant Breeding laboratory, and the Molecular Biology Laboratory of the Indonesian Vegetables Research Institute located in Lembang, West Java. Additionally, some of the research activities were carried out at the Molecular Biology Laboratory of the Indonesian Research Institute for Biotechnology and Bioprocess in Bogor, West Java.

2.2. Treatment
The research treatment involved three chili varieties (i.e. RK-5, F1, and RK-3) with different level of resistance to fruit fly from previous research [15]. RK-5 was a variety of red chili with susceptibility to fruit fly infestation, whereas RK-3 was a wrinkled variety of chili resistant to fruit fly infestation, and F1 was the first generation of crossing between RK-5 and RK-3 with moderate resistance to fruit fly infestation.

2.3. The Phenotypic Response
The phenotypic/physical active responses of chili fruit to fruit fly infestation were measured by using the fitness index, pupae weight (mg) per weight (g) of chili fruits, and fruit rot (%). The three treatments in this research involved the artificial infestation of the fruit fly eggs according to [5]. Firstly, the chili pericarp was thinly sliced. Secondly, the fruit fly eggs were inserted into the chili fruits. The egg-contained chili fruits were then incubated for 30 days. Afterwards, the observation was done to obtain data on the number of pupae, the pupa weight (mg), the duration from larvae to pupae (days), as well as the duration from pupae to imago (days).
2.4. The Genotypic Response

2.4.1. RNA Isolation and cDNA construction. In the treatment, RNA was isolated from RK-5, F1_3, and RK-3 fruits under both the healthy and infested conditions. The procedure of infesting the eggs of fruit flies into the chili fruits were based on [16]. The total number of RNA isolation products used in this research was six with three replications. Furthermore, the RNA isolation was done by using GeneAll RiboEx Total RNA Solution Kit with the instructions from the manufacturer. The RNA was changed into cDNA to be further amplified by the PCR method. Then, the cDNA synthesis was conducted by employing the Reverse Transcriptase PCR (RT-PCR) thermal cycler C1000 (Biorad) Kit. This cDNA synthesis program included annealing (37°C, 30 s); cDNA synthesis (48°C 4 min); melting secondary structure (55°C, 30 s) and heat inactive (95°C, 5 min) as many as 12 cycles.

2.4.2. RT-PCR Program. Two types of primary were used in this research, namely the targeted gene (CaRGA2) and the housekeeping gene (Actin). The oligonucleotide sequence of the targeted gene comprised of CaRGA2-F (5’-TGCTAGGCCGGAACAGGTATG-3’) and CaRGA2-R (5’-CAAGCCGATGTTGTTAGAACAG-3’) with the amplion fragment size of 486 bp. Meanwhile, the oligonucleotide sequence of the housekeeping gene consisted of Actin-F (5’-ACTCTTAATCAATCCCTC-3’) and Actin-R (5’GCACTGTATGACTGACACC-3’). Moreover, the cDNA RT-PCR program included pre-denaturation (95°C , 1 min); denaturation (95°C, 15 s); annealing (56°C, 15 s), extension (72°C, 10 s) and post-extension (72°C, 20 s) as many as 35 cycles.

2.5. Data analysis
The analysis of RT-PCR data in this research was conducted by employing the Livak method [17] while the CaRGA2 gene expression was analyzed based on the number of copies obtained from the ΔC\text{t} calculation. Furthermore, the statistical data analysis in this research was the Kruskal-Wallis Test using the Past program [18].

3. Results and discussion
The statistical results using the Kruskal-Wallis test showed that the values of fruit fly fitness index for both the RK-3 and F1_3 chilies were significantly lower than that of the RK-5 chilies (see Table 1). The fitness index itself is one of the parameters used to assess the survival of an organism [16, 19]. Therefore, these results indicated that the survival rates of fruit flies on the RK-3 and F1_3 chilies were lower than that of the RK-5 chilies. Moreover, differences in the survival of fruit flies among the chili varieties may indicate differences in active responses of these varieties. Several studies have identified the active response of the host plant as antibiosis, a mechanism that works upon being colonized by insects, which can eventually inhibit larval development [20-22]. In this study, the antibiosis of the RK-3 and F1_3 chilies were able to reduce the fitness index of fruit fly larvae.

| Varieties | Fitness Index | Total pupa weight (mg) | Fruit rot (%) |
|-----------|---------------|------------------------|---------------|
| RK-3      | 0.09 a        | 1.27 a                 | 4.74 a        |
| F1_3      | 0.08 a        | 2.48 a                 | 23.20 b       |
| RK-5      | 2.61 b        | 11.21 b                | 45.90 c       |

Furthermore, the statistical results of the Kruskal-Wallis test in Table 1 illustrated that the pupa weights of fruit flies harvested from the RK-3 and F1_3 fruits were significantly lighter than that of the RK-5 chilies. The lighter weight of the pupae of the RK-3 and F1_3 fruits indicated that the RK-3 and F1_3 fruits were less supportive of larval growth and development (i.e. the phase before becoming pupa). The antibiosis mechanism might occur in the forms of limited nutritional factors or the presence of toxic metabolite factors [20, 22, 23], therefore further in-depth research regarding the form of antibiosis resulted from this study would be deemed necessary. Moreover, pupae with the maximum
size were produced from the RK-5 chilies. This indicated that the larvae could reach the maximum growth and development, just as [24] reported previously in their study that in Ceratitis capitata, the maximum pupa size would result in an imago that was more adaptable for the next life cycle.

Previous data on the fitness index and pupa weight illustrated the active response of chilies to fruit fly infestations that were physically visible (i.e. phenotypic). Genotypically, the results from three different varieties of chilies in this study demonstrated that there were differences in the relative expression of chili defense genes against biotic stress (CaRGA2) after being infested with fruit fly eggs. Moreover, the RT-PCR results displayed the differences in the expression of CaRGA2 gene in RK-3, F1.5,3, and RK-5 (Table 2). The mean \( C_T \) values of RK-3 and F1.5,3 were higher than that of RK-5. As Livak and Schmittgen [17] stated that a high \( C_T \) value of a gene might indicate the very low or unexpressed genes, these results revealed that in the infested conditions of fruit fly eggs, the CaRGA2 gene was more expressed in RK-5 than it was in RK-3 and F1.5,3.

### Table 2. The genotypic response of chili varieties with different level of resistance to fruit fly

| Varieties | \( C_T \) Before Oviposition | \( C_T \) After Oviposition | \( \Delta \Delta C_T \) | Relative Expression of CaRGA2 |
|-----------|-------------------------------|-----------------------------|----------------------|-------------------------------|
| RK-3      | 2.73                          | 16.74                       | 14.01                | 0.00 a                        |
| F1.5,3    | 2.02                          | 16.14                       | 12.12                | 0.00 a                        |
| RK-5      | 1.84                          | 3.57                        | 1.72                 | 0.32 b                        |

Zhang et al. [9] and Esyanti et al. [14] reported that the presence of pathogenic fungi caused the CaRGA2 gene to be expressed. In this study, the rotting process of chilies after oviposition was relatively slower or even it did not occur in RK-3 and F1.5,3 (Table 1). On the contrary, it was very fast in RK-5. These results reinforced the assumption of [10], which stated that wounds due to insect oviposition on host plant fruits could enhance secondary infections caused by microorganisms. In addition, the fruit rot that happened to RK-5 chilies was due to the presence of pathogenic fungi and subsequently caused the CaRGA2 gene to be expressed, as Zhang et al. [9] further mentioned that the expression of the CaRGA2 gene would increase along with the increasing number of pathogenic fungal spores on the fruit of the host plant.

Eventually, the results of this study may lead to develop protocols to be used for selection resistance to fruit fly in the plant breeding programs. Selection of varieties resistant and susceptible to fruit flies can be carried out more effectively using a gene expression approach. Under conditions of stress by biotics (in this case the oviposition of fruit flies), susceptible varieties can be detected by the rise on defense genes expression as a form of induced defense, whereas resistant varieties already have their own defenses using nutritional attributes or synthesizing toxic metabolites. Hereafter, further studies are required in order to fulfill the finding of this study, such as to identify nutrient content and metabolite compounds in resistant varieties of chilies associated with inhibition of fruit fly larvae and pupae size, as well as to identify many other induced or expressed defense genes during biotic stress on chili.

**Author’s contributorships**
All authors contributed equally to this work.

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