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Research Article

THE ROLE OF CHITINASE IN THE PATHOGENICITY OF LECANICILLIUM LECANII BASED ON GENETIC – ENZYMATIC ANALYSIS AND BIOASSAY AGAINST NYMPH POD SUCKING BUG *RIPTORTUS LINEARIS*

Yayuk Mulyati[1*], Toto Himawan[1], Estri Laras A[2]. and A. Latief Abadi[1]

1. Department of Plant Pest and Desease, Faculty of Agriculture, Brawijaya University, Indonesia.
2. Department of Biology, Faculty of Mathematic and Natural Science, Brawijaya University, Indonesia.

***Corresponding Author: Email yayukmulyati1982@gmail.com**

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ABSTRACT

Objectives: The research aimed to evaluate the role of chitinase in the pathogenicity of Lecanicillium lecanii based on comprehensive analysis of mutation occurences in the partial sequence of Chit1 gene, comparison and correlation of chitinase production and pathogenicity between wild type and mutant.
Methods: Mutation was conducted using ultraviolet-C radiation (UV-C) with the period of exposure of 0, 2 (UV-C2), and 4 (UV-C4) hours. Genomic DNA was isolated using NucleoSpin Plant II kit, and then was PCR-sequenced, and the sequencing result of wild type and mutant was analysis for their alignment. Chitinase production were analyzed using Schales methods. Pathogenicity of wild type and mutant tested against nymphs pod sucking bug Riptortus linearis in laboratory conditions.
Results and Conclusions: The results showed that UV radiation caused mutations of partial sequence of Chit1 genes. The mutation occurences in the mutant UV-C2 is 2.47 times higher than mutant UV-C4. Wild type and mutant showed significantly different chitinase secretion. Chitinase production in mutant UV-C2 and mutant UV-C4 were 1.003 and 1.012 fold higher than wild type, respectively. The higher ability of mutant to produce chitinase compared to wild type was not followed by their pathogenicity. The mortality of R. linearis nymph was higher when it was infected by wild type compared to mutant. The wild type and mutant showed no difference in their pathogenicity in the sixth and tenth days of observation. Evaluation of the overall findings of these study lead to the conclusion that chitinase is not the most important hydrolytic enzyme in the pathogenicity of L. lecanii.
Keywords: ultraviolet-C radiation (UV-C), Chit1 gene, mutation, chitinase production, ability to infect.

INTRODUCTION

Lecanicillium lecanii (Zimm.) (Viegas) Zare & Gams is one of entomopathogenic fungi that already known for its potential to control some insect species [1]; including pod sucking bug, *Riptortus linearis*. *L. lecanii* was reported to have the highest potency in controlling *R. linearis* among other fungus such as *Beauveria bassiana* (Balsamo) Vuillemin, *Nomuraea rileyi* (Yasuda), *Paecilomyces fumosoroseus* (Samson), dan *Metarhizium anisopliae* (Metschnikoff) Sorokin (=*flavoviride*). The potency of *L. lecanii* in controlling *R. linearis* is also due to its ability to infect all stadia these pests [2].

The prospect of *L. lecanii* as a potential biological control is associated with its ability to produce hydrolytic enzymes. The hydrolytic enzyme acts to degrade insect cuticle which is the first barrier against entomopathogenic fungi [3]. Chitinase is one of hydrolytic enzyme that important in *L. lecanii*. Research on three strains of *L. lecanii* showed that strains which were able to produce the highest chitinase was a strain that has the highest infection against green peach aphids *Myzus persicae* [4].

Until now, most chitinase research aimed to examine the effect of various physical factors on the production of

chitinase. Some physical factors examined include pH, temperature, period of incubation, the growth substrate, substrate concentration and the others. However, a study evaluating the effect of ultraviolet (UV) radiation to the chitinase gene as well as chitinase enzyme of entomopathogenic fungi, especially in *L. lecanii* was very rarely done.

UV radiation is the main physical factors which become obstacles when the fungus is applied in the field. Generally, a few hours of direct exposure to UV radiation can cause DNA mutations. DNA mutation causes the inactivation of conidia, delay germination, even cell death [5]. These conditions affect the effectiveness of entomopathogenic fungi as biopesticide [6-7]. In this research, UV radiation used for inducing mutation was UV-C. UV-C has a wavelength range of 250-254 nm which will maximally absorb by nucleic acids. Therefore, UV-C is often referred to ultraviolet biologically-effective [8]. Many references mentioned that UV radiation is most harmful mutagen to fungi. However, what genes which were mutated and what kind of mutation that occurs were almost never explained further [9]. *Chit*1gene is an acidic chitinase gene 1891 bp length. These gene has been successfully sequenced in 2005 [10]. Studies on the effect of UV-C radiation on the *Chit*1 gene still has not been done to date. In this study, mutation due to UV-C radiation was assessed against a gene encoding chitinase *L. lecanii*, i.e. chitinase (*Chit*1) gene.

The occurrence of mutations was confirmed by comparing the amount of the secretion of chitinase produced by irradiated (mutant) and not irradiated (wild type) *L. lecanii*. The pathogenicity of wild type and mutant *L. lecanii* was tested against the nymphs of *R. linearis*. Overall findings of this study were expected to provide information related to the role of chitinase in pathogenicity *L. lecanii*.

MATERIALS AND METHODS

Fungal strains and inoculum preparation

L. lecanii was obtained from Indonesian Legumes and Tuber Crops Research Institute. The microorganism was maintained on potato dextrose agar slants, sub-cultured regularly every 1 month and incubated at room temperature for seven days. Conidia were harvested with distilled water and sieved through filter paper into sterile vials. Conidia were counted using haemocytometer to calibrate a suspension of 1 x 10^6 conidia/mL. The spore suspension was used as inoculum for UV-C radiation treatment.

UV C radiation treatment

UV-C irradiation procedure refers to Chelico *et al.* with modification [7]. Erlenmeyer flask 250 mL containing 30 mL of conidial suspension at concentration of 1 x 10^6 was placed under an ultraviolet lamp (Germacidal lamp, G30T8 6G, 30W 254 nm). The distance between the lamp with the erlenmeyer flask was 50 cm. UV lamp was turned on for 1 hour prior to radiation treatment to maintain the stability of UV radiation. During irradiation, the suspension was stirred continuously with a magnetic stirrer and stir bar at 250 rpm. Conidia suspension was irradiated for 0, 2, and 4 hours. Conidia suspensions that were subjected to UV radiation was used for molecular analysis, chitinase activity analysis, and pathogenicity test on the nymphs of *R. linearis*.

Effect of UV-C irradiation to the mutation occurance of partial Chitinase (*Chit*1) gene of *L. lecanii*

DNA was isolated from the fungus which was previously grown on PDA. The plates with 7 mm diffusion holes were innoculated with 65 µl conidial suspension that have been irradiated or non-irradiated. The fungus was incubated at room temperature in dark conditions. DNA of fungi isolated in 5 days after incubation. DNA isolation procedures referred to the NucleoSPin Plant II Kit (Macherey Nagel).

*Chit*1 gene sequences were amplified referring to Chit1 gene sequences from *L. lecanii* strains DAOM 175 104, GenBank: AY705924.1 (http://www.ncbi.nlm.nih.gov/nuccore/AY705924.1). Primer sequences used to amplify the *Chit*1 gene were Forward primer: 5'-ATCAAAGAGCGCGGACTAGG-3', reverse primer: 5'-GTACGCCAACGAGCATGAA C-3' which produced amplification fragment of approximatelly 511 bp. The partial sequence of *Chit*1 gene was amplified in a TAKARA PCR Thermal Cycles (Japan).

PCR reaction mixture contained 10 µL of 2X PCR Master Mix solution (i Taq TM), 1 µL of DNA template, 1 µL of 10 pmol/µL forward and reverse primers, and 7 µL of distilled water to make 20 µL total reaction volume. The amplification condition were: 94°C for 2 min initial denaturation, followed by 30 cycles of 94°C for 20 s denaturation, 57°C for 10 s annealing, and 72°C for 20 s extention. The final extention was conducted at 72°C for 5 min. PCR result were then

checked in 1.5% agarose and visualized by using Geldoc Major Science UVDI-254/365, Taiwan.

Mutation analysis of partial *Chit*1 gene of *L. lecanii*

The expected band of partial sequence of *Chit*1 was excised and extracted as a pre-treatment prior the sequencing process. Sequencing protocol refers to BigDye® Terminator v3.1 Cycle Sequencing Kit. The partial sequence of *Chit*1 gene was sequenced using ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA. Homology analysis was conducted between *Chit*1 gene partial sequences that have been irradiated for 2 and 4 hours compared to the wild type chitinase gene sequences using Basic Local Alignment Search Tool (BLASTn) program. The percentage of mutation occurrence and type of mutations in each treatment period of UV-C irradiation were analyzed descriptively.

Effect of UV-C radiation to the chitinase production of *L. lecanii*

The composition of the basal medium used to grow the fungus is as follows (g/100 mL): NAD 0.5, K_2HPO_4, 0.1; urea, 0.3; MgSO4, 0.05; FeSO4, 0.01; yeast extract, 0.01. Erlenmeyer flask containing 35 ml medium were sterilized (121°C for 15 min). The flask were inoculated with 1 ml of 1 x 10^6 conidia/mL that have been irradiated and non-irradiated. Culture were incubated at room temperature for 5 days on rotary shaker. Thereafter, culture were centrifugated at 3000 rpm for 10 min in a cooling centrifuge (4°C). The clear supernatant was considered as crude enzyme to assay chitinase activity [11]. The experimental design was a group randomized design with 6 replications.

Chitinase activity assay

Chitinase activity was analyzed by using colloidal chitin as substrate. The colloidal chitin was prepared according to the method described by Roberts and Selintrenikoff with modification [12]. Ten grams of chitin powder from shrimp shell (Sigma, USA) was slowly added into 200 ml concentrated HCl with vigorous stirring for 1 hour and kept overnight at 4°C. The solution was filtered through glasswool. The resulting filtrate was then added with 100 ml of distilled water and neutralized with NaOH 12N. The chitin suspension was centrifugated at 3000 rpm for 60 minutes at 4°C. The precipitate added with distilled water, stirred to dissolved residual salt, and then centrifugated again at 3000 rpm for 60 min at 4°C. The precipitate (colloidal chitin) stored at 4°C until used.

Analyzes were conducted on the fifth day after incubation. Cultures were centrifugated at 3000 rpm for 10 min in a cooling centrifuge (4°C). The clear supernatant was considered as crude enzyme to assay chitinase activity[11]. Chitinase activity was measured by the equivalent amount of N-acetylglucosamine released from colloidal chitin by following the method of Spindler [13]. The reaction mixture consisted of 0.45 mL of crude enzyme and 0.9 mL 1% (w/v) colloidal chitin in phosphat buffer (0.05 M, pH 5.6) and incubated at 37°C for 30 min. The reaction was stopped by heating the solution in boiling water for 10 min and then cooled. Thereafter, the reaction mixture was centrifuged at 3000 rpm for 5 min. To the supernatant, 3 ml Schales reagents and 1.5 ml distilled water was added, and then heated in boiling water for 10 minutes for colour development. After cooling, the developed colour, as indication of the quantity of released N-acetylglucosamine (NAD), was read at 420 nm in Ganesys 10UV Spectrophotometer.

Standard graph was prepared to convert the absorbance values to micromoles of N-acetylglucosamine liberated from colloidal chitin. The data obtained from the experiments were subjected to analysis of variance (ANOVA) and the means showing statistical significance were compared by Least Significant Different (LSD) test.

Pathogenicity assay of mutant and wild type *L. lecanii* to the nymph *R. linearis*

Fungi grown on solid media with the following composition: 0.5 g/100 mL NAD, 0.1 g/100 mL K_2HPO_4, 0.3g/100 mL urea,; 0.05g/100 mL MgSO4,; 0.01g/100 mL FeSO4,; 0.01 g/100 mL yeast extract,; 1.5 g/100 mL bacto agar. Media were auctoclaved at 121°C for 15 min and 15 ml of each medium was poured into 9 cm Petri dishes. The plates with diffusion hole 7 mm were inoculated with 65 µl irradiated or not irradiated conidial suspension. The cultures were incubated for 5 days at room temperature with dark conditions with four replications.

The conidia of *L. Lecanii* mutant and wild type were harvested with distilled water and sieved through filter paper into sterile vials. Conidia were counted using haemocytometer to calibrate a suspension of 1 x 10^8

conidia/mL. As much as 20 first-instar nymphs of *R. linearis* was sprayed at the rate of 3 mL/treatment and then placed into each container (diameter = 10 cm, height = 26 cm). Each container supplied with 2 pieces of fresh long beans. Feed was replaced every two days. The mortality of the nymph of *R. Linearis was* recorded on days 3, 6, and 10 after application. Data was analyzed by two way analysis of variance and the means which were showing statistical significance compared by LSD test.

RESULTS

Amplification of partial sequence chitinase (*Chit*1) gene of *L. lecanii*

Following figure (Fig. 1) shows the results of amplification of partial sequence of *L. lecanii* wild type and mutant *Chit*1 gene. The expected bands of all treatments were in the same position, which is about 511 bp.

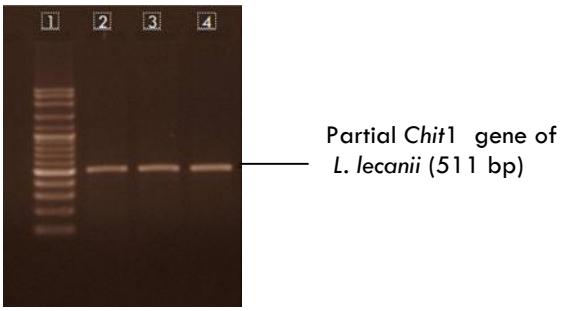

Fig. 1: Visualization of partial *Chit*1 gene amplification results by 1.5% agarose gel electrophoresis. Well 1: DNA ladder of 100 bp, well 2: partial *Chit*1 gene of wild type *L. lecanii*, well 3 and 4: partial *Chit*1 gene of *L. lecanii* which have been irradiated for 2 (mutant UV-C2) and 4 hours (mutant UV-C4).

Result of BLAST analysis

Sequencing analysis resulted in 424 bp readable nucleotide from 511 bp *Chit*1 gene partial sequence of mutant UV-C2 (Fig. 2); while on mutant UV-C4, the readable nucleotides was 420 bp (Fig. 3). The percentage of mutations in the mutant UV-C2 was 2.47 fold to the mutant UV-C4. Mutant UV-C2 showed 37 mutated nucleotide bases, consist of 13 transition mutation and 24 transversion mutations. Mutant UV-C4 showed 15 mutated nucleotide bases, consist of 6 transition mutations and 9 transversion mutation.

Effect of UV-C radiation to the chitinase production of *L. lecanii*

UV radiation treatment caused changes in *L. lecanii* chitinase production. Analysis of variance result showed that the treatment given was significantly different to the chitinase production between mutant and wild type ($F_{2,10}$ = 110.60, P = 0.01). Chitinase production of mutant UV-C2 and UV-C4 was 1.003 and 1.012 fold to wild type respectively (Fig. 4).

Pathogenicity assay of mutant and wild type *L. lecanii* to the nymph *R. linearis*

Two way analysis of variance results showed that UV radiation and day observation influence the pathogenicity of *L. lecanii* significantly ($F_{2,10}$ = 3.54, P = 0.05). The pathogenicity of *L. lecanii* mutant and wild type at 3, 6, and 10 days after inoculation is shown in Fig. 5. At 3 days after inoculation, the wild type showed the highest pathogenicity, followed by mutant UV-C4 and mutant UV-C2. The pathogenicity of wild type and mutant showed no difference on 6 and 10 days after inoculation.

DISCUSSION

Effect of UV-C radiation on mutation occurrence in partial sequence of *Chit*1 Gene

Alignment of *Chit*1 gene partial sequence between wild type to mutant UV-C2 and wild type to mutant UV-C4 indicate that the period of UV radiation affects the frequency of the mutation. The mutation occurrences in the chitinase gene as a result of UV radiation has been reported previously by Patil although there was no evidence of those mutations [14]. Different responses as a result of UV-C radiation was common, and related with the mutation characteristics as genetic process, i.e. accidental and not directed [15]. Transversion and transition is spontaneous mutation with the frequency of occurrence is higher than other types of mutations [16].

Effect of UV-C radiation to the chitinase production of *L. lecanii*

UV radiation was able to change the chitinase production of *L. lecanii* [17-18]. The ability of the mutant to produce an enzyme that is higher than wild type after exposure to UV radiation were also reported [14, 19-20]. The mutant ability to produce chitinase was higher than the wild type, which is possible as a process of adaptation of the fungi; altthough the mutation is not directed genetic process [15]. Mutations believed to be an adaptation process in order to optimize the utilization of nutrients in the surrounding environment [9].

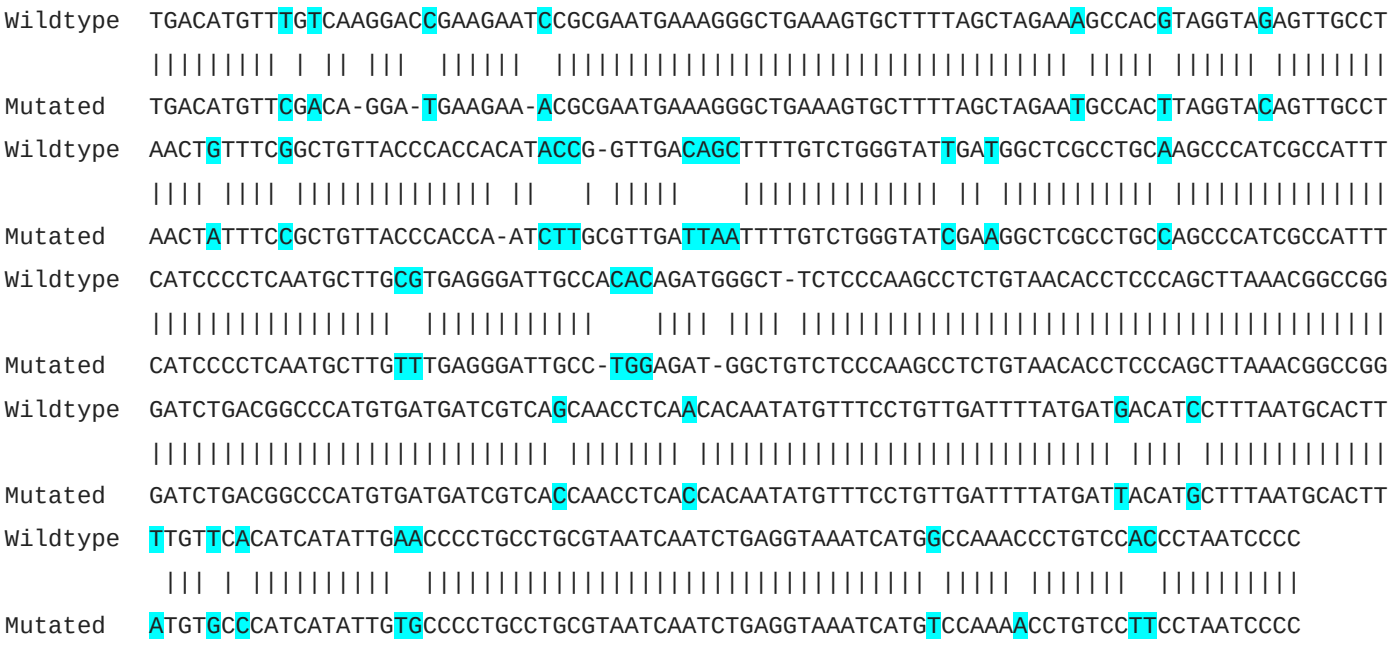

Fig. 2: Aligment of partial sequence *Chit*1 gene between wildtype and mutant UV-C2

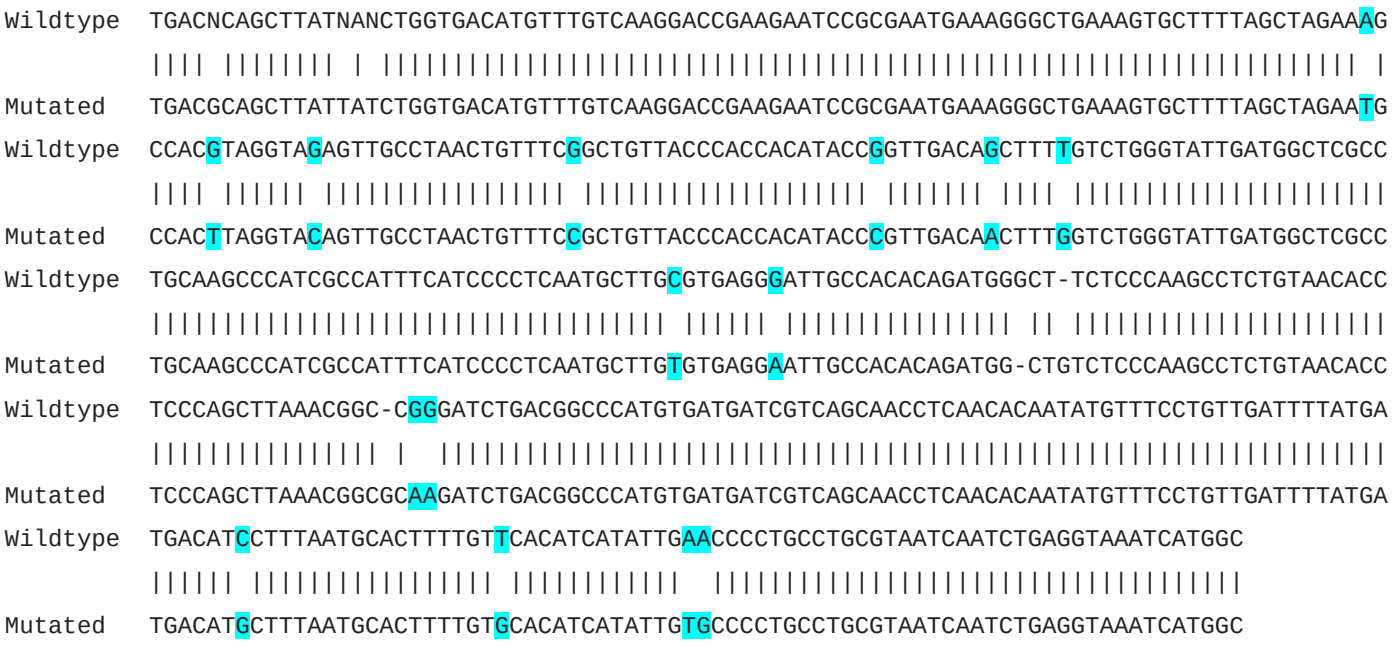

Fig. 3: Aligment of partial sequence *Chit*1 gene between wildtype and mutant UV-C4

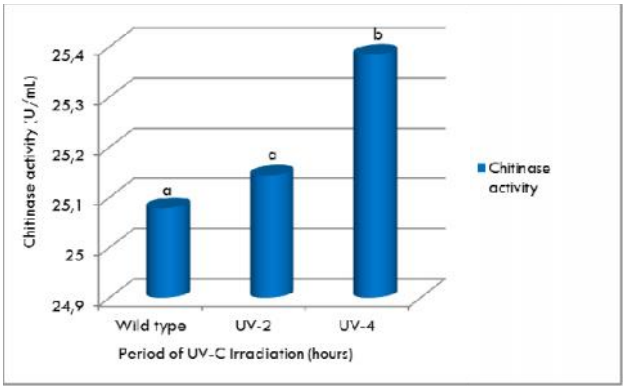

Fig. 4: Comparison of chitinase production between wild type and mutant *L. Lecanii*

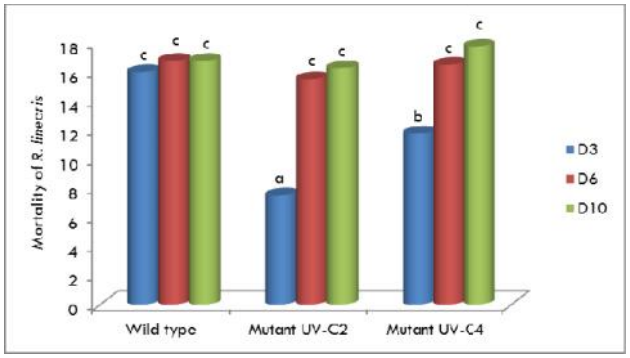

Fig. 5: Comparison of pathogenicity of wild-type and mutant *L. lecanii* on 3 (D3), 6 (D6), and 10 (D10) day after inoculation

Pathogenicity assay of mutant and wild type *L. lecanii* to the nymph *R. linearis*

The ability of mutants to infect *the nymph R. Linearis was* lower than the wild type based on the third day observation and then increased and showed no difference with the wild type on the sixth and tenth day observations. These indicate that there has been a temporary inactivation on mutant [21]. Time span of 11 days from radiation treatment to the second observation was a long period. During that time, it was possible that stabilisation of genetic changes or DNA repair to occur [7, 22]. The low pathogenicity of mutant compared to the wild type was agree with Sahab findings which found that the mutant *B. bassiana* has a lower infection compared to wildtype against *Ostrinia nubilalis*, *Chilo Agamemnon*, and *Sesamia cretica* of corn insect pests [9]. However, the studies were not mentioned how many days after inoculation did the observations were conducted and no explanation for the increasing fungal pathogenicity. Based on the overall findings of this research; the period of UV exposure for 2 and 4 hours was sufficient to change the *Chit*1 gene partial sequence, chitinase production, and pathogenicity of *L. lecanii*. Prolonged exposure to UV radiation for 2 and 4 hours were able to induce mutation on *L. lecanii*. The mutant showed different expression based on the ability to produce chitinase. Mutant *L. lecanii* can produce chitinase more than wild type, but the mortality observations of nymph *R. linearis* on the third day showed that the wild type was more pathogenic than the mutant. The non linear result between chitinase production with the pathogenicity of wild type and mutant has been reported previously [19].

Additional information draw from these results that the chitinase was not the most and only important enzyme among other degradative enzymes that determine the pathogenicity of *L. lecanii*. However, studies on the effects of UV radiation on the other hydrolytic enzymeis expected to produce definitive answers related the regulation mechanism of hydrolytic enzymes in the insect cuticle infections.

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