Entomopathogenicity of *Simplicillium lanosoniveum* CG888 on Mortality of *Callosobruchus maculatus* F. in vitro

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Abstract. Research regarding *Simplicillium lanosoniveum* as an entomopathogen is still limited. The entomopathogenicity of *Simplicillium lanosoniveum* CG888 with three different conidia density treatments, namely $1x10^6$/ml, $1x10^7$/ml, and $1x10^8$/ml, was tested on *Callosobruchus maculatus* F. imago in the laboratory, incubated at 26-27°C, RH 70% and in the dark conditions. Based on the effect of conidia density, *S. lanosoniveum* CG888 at a density of $1x10^8$ conidia/ml caused greater mortality of *C. maculatus* F, reaching 51.65% compared to other treatments and control. The influence of the length of time after inoculation was that there was 60.83% *C. maculatus* F. death on the 11th day. It was higher than the result on 3, 5, 7 and 9 days later. There was a positive interaction that the mortality of *C. maculatus* F was influenced by the density of *S. lanosoniveum* CG888 conidia and the period of days after inoculation. Among these treatments, *S. lanosoniveum* CG888 with $1x10^8$/ml caused mortality of 86,7% in *C. maculatus* on day 11 after inoculation. The lowest mortality of *C. maculatus* F. was in the density of $1x10^6$ conidia/ml of 70%. These findings indicate that *S. lanosoniveum* CG888 can be used to control *C. maculatus* F. safely and reduce the use of chemicals in soybean storage.

1 Introduction

Callosobruchus maculatus Fabricius is a primary pest that attacks peanut commodities in storage. It causes yield losses in soybeans (10 – 15%) [1] and green beans (50-70%) [2]. This pest puts the eggs in the seeds, and at the larval stage, it holes in the seeds to eat the structure inside. The attack results in the seeds cracking, which can cause damage to the seed structure and reduce its viability. The most common and efficient pest control is the use of synthetic pesticides. Its use on agricultural commodities has a significant effect on increasing the quantity and quality of production to meet the demands of food and industrial needs. However, exposure to pesticides causes residual effects on agricultural commodities. Based on monitoring results [3] of research on synthetic pesticide residues in soybeans in Indonesia,

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there were active ingredients Lindane, Carbofuran, Diazinon, and even DDT detected in soybean commodities in the period 1992 - 1997. This could disrupt the quality of the commodity in supporting food safety in Indonesia.

Entomopathogenic fungi are an alternative for warehouse pest control to reduce the use of synthetic pesticides [4, 5]. One of them is *Simplicillium lanosoniveum.* Research regarding this fungus [6] as an entomopathogenic is still limited [7-11]. Based on the above fact, research was carried out regarding the effect of the application of *S. lanosoniveum* CG888 [13] on the soybean warehouse pest *C. maculatus* F. mortality at different conidia densities. Hopefully, this research can become a reference in developing the biodiversity of entomopathogenic fungi, applying effective warehouse pest control and obtaining soybean commodities that are safe from pesticides.

2 Materials and methods

2.1 Determination of conidia density and viability percentage of fungi

At first, Potato Dextrose Agar (PDA) brand Ltd Darmstad Germany as much 19.5 grams was dissolved in 500 ml of distilled water. The solution was heated to boiling and sterilized in an autoclave at 121oC for 15 minutes. Then, it was placed in laminar air flow, and under sterile conditions, 1% chlorofemicol was added to it. After that, the solution was poured 10 ml into a 9 cm^3 petri dish, then waited until the media solidified and was ready to be inoculated.

Simplicillium lanosoniveum CG888 was diluted to determine conidia density. The fungus culture was made into a suspension with 10 ml distilled water in a test tube. Then, the suspension was homogenized with a magnetic stirrer for 5 seconds at 50 rpm. The suspension was taken at 0.2 ml using a 10 ml Dragon Lab brand micropipette. Then it was dropped into a German Neubauer brand haemocytometer. Density counts were carried out under an Olympus cx31 microscope (Olympus Corporation, Tokyo, Japan) by calculating the density of conidia/ml in each counting box. Conidia density was calculated until a conidia density of 108/ml was obtained using the following formula [13].

$$s = \frac{x}{Ltd}\left(10^3\right) \quad (1)$$

Information: S = density conidia/ml; x = the number of conidia in boxes a, b, c, d, e; L = area of calculating box 0.2 mm^2 (0.004 mm^2 x 5 squares); t = count depth of 0.1 mm; d = dilution factor

To determine the viability of *S. lanosoniveum* conidia, PDA media in the petri dish was cut to a diameter of 7 mm using a cork drill. Then, the piece is placed on an object glass. Each object glass contained three pieces of PDA media as three replicates. Next, a conidia suspension with a density of 10^8 conidia/ml was dripped onto each piece as much as 1 ml. Each piece of PDA media was covered with a cover glass. Petri dishes were prepared and filled with cotton rolls weighing 0.45 grams and moistened with 5 ml of distilled water. The object glass was placed in a petri dish and incubated for 24 hours at room temperature. Conidia were observed under a microscope at 400x magnification. Conidia viability is determined if the conidia germinate with the sprout size being two times the length of the conidia [14], which is expressed in the percentage of conidia germination [15].

2.2 Entomopathogenicity test

Callosobruchus maculatus F. used for entomopathogenicity testing is breeding results in the adult stage, ranges from 4 – 5 days and is in healthy or active condition. *Simplicillium lanosoniveum* CG888 with a conidia density of 108/ml was diluted to test its pathogenicity on C. maculatus F. The test was based on conidia density and time after inoculation on C. maculatus F mortality. Then, 10 ml of the solution was taken using a micropipette, put into a new test tube filled with distilled water to 100 ml, and stirred. Next, the same procedure was done until the conidia density was obtained according to the treatment. The treatment factor tested was the conidia density of the fungus, which consisted of four levels, namely: S0 = 0/ distilled water (control), conidia density of *S. lanosoniveum* CG888 respectively S1 = 106 conidia/ml, S2 = 107 conidia/ml, S3 = 108 conidia/ ml. Each treatment used three replications, consisting of 20 adult individuals/replication.

The virulence test of *S. lanosoniveum* CG888 to cause mortality in *C. maculatus* F used a modified Shimazu technique [15]. One millilitre of conidia suspension from each conidia density treatment was spread onto a PDA in a petri dish and incubated for 14 days at 26–27°C. The fungi that have grown in petri dishes from the incubation results are used for virulence testing. Twenty imagoes were placed into each petri dish. They were allowed to activate the fungus for 12 hours. After that, it was transferred to a sterile petri dish containing only soybean seed fragments. The soybeans used have been soaked briefly in distilled water and then air-dried. Incubation was carried out at room temperature 26–27°C, RH 70% and in dark conditions. Observation of *C. maculatus* F. mortality was carried out every day from the time of application until two weeks later. The observation includes the following variables: a) symptoms of *S. lanosoniveum* infection against *C. maculatus* F., and b) percentage of pest mortality [16]. The experimental design of this research used a completely randomized design. Data were analyzed using two-way ANOVA at 95% level and continued with the BNT test ($p > 0.05$) if there were differences between treatments.

3 Result and discussion

Application of *S. lanosoniveum* CG888 (Fig. 1) caused mortality in *C. maculatus* F. The symptoms of infection appeared 4-6 days after inoculation, reached its peak around the 7th day, and then mycelia began to appear on his body (Fig. 1d). It is suspected that the penetration of conidia that sticks to the insect's cuticle infected the body and caused mortality. Insects that died because of being infected by *S. lanosoniveum* CG888 were shown with a darker abdominal colour and white mycelium covering almost the entire lower body surface. On the 14th day, the insects become mummified and dry.

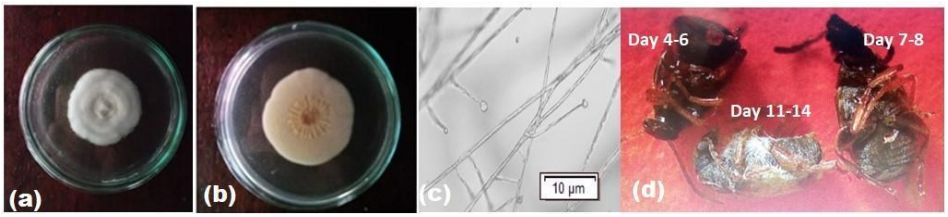

Fig. 1. Surface appearance of *Simplicillium lanosoniveum* CG888 aged 25 days (a) top, (b) bottom, (c) mycelia, (d) Symptoms *C. maculatus* F. is infected by *S. lanosoniveum* CG888.

Mechanical transmission of *S. lanosoniveum* CG888 to *C. maculatus* F. occurred when conidia made contact with the abdominal organs, which are the soft body parts of the insect. When contact occurs, the conidia attach to the abdomen, then germinate and penetrate the

cuticle. Hyphae infect the insect's body through the cuticular surface and natural holes. The bodies of insects infected with the fungus look dry. This condition showed that the fungus has developed and taken nutrients from the insect's body for reproduction [17].

Table 1 showed that based on the effect of conidia density, a concentration of 10^8 conidia/ml caused greater mortality of *C. maculatus* F., reaching 51.65% compared to the other two treatments and the control. Meanwhile, based on the influence of the length of time after inoculation, on the 11th day, there were around 60.83% *C. maculatus* F. deaths. It was higher than the result on 3, 5, 7 and 9 days later.

Table 1. The effect of *S. lanosoniveum* CG888 inoculation treatment based on conidia density and time after inoculation on the mortality of *C. maculatus* F was tested

Densities	Mortality (%)	Time (day)	Mortality (%)	Interaction
Control (S0)	4.00[a]	3	3.75[a]	
1 x 10^6 (S1)	29.65 [b]	5	13.75[b]	
1 x 10^7 (S2)	35.67[bc]	7	27.08[c]	
1 x 10^8 (S3)	51.65 [d]	9	45.83[d]	
		11	60.83[e]	
df	3		4	12
SS	706.45		1038.10	233.63
MS	235.483		259.525	19.469
F-value	166.22		183.19	13.74
Pr (>F)	0		0	0

Note: Numbers followed by the same letter indicate that they are not significantly different in the 5% BNT test

There was a positive interaction that the mortality of *C. maculatus* F was influenced by the density of *S. lanosoniveum* CG888 conidia and the period of days after inoculation. (Fig. 2). There was a significant increase in the number of individual deaths at a conidia density of 1 x 10^8/ml, along with increasing days after inoculation. Conidia density influences insect mortality rates. The conidia density of 1 x 10^8/ml caused a greater increase in adult *C. maculatus* F mortality compared to the other two treatments. High conidia density increases the potential for high fungal pathogenicity as well. This condition causes the applied insect to be exposed to more conidia, so the possibility of its body being infected by the fungus is greater.

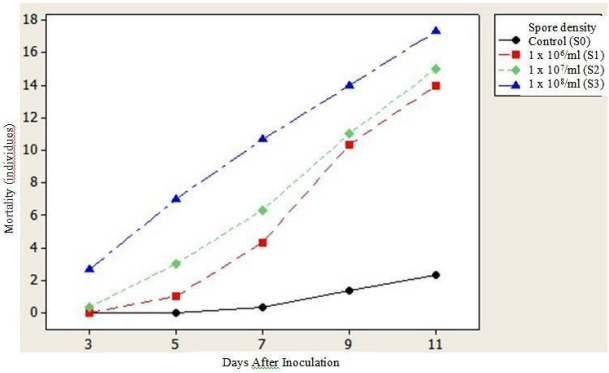

Fig. 2. Diagram of interaction for mortality of *C. maculatus* F. on conidia density and day after inoculation with *S. lanosoniveum* CG888.

Temperature and humidity during incubation are 26 – 27°C and RH 70%, with dark conditions. These conditions are suitable to support *S. lanosoniveum* in the process of infection of the insect's body [18]. In the results of the application of the fungus *Lecanicillium muscarium* (972) with a conidia density of 1 x 10^7/ml against *C. maculatus,* the highest mortality rate ranged from 84 - 100 per cent at a temperature of 26ºC and RH 70% [4]. The viability of *S. lanosoniveum* CG888 conidia, which is in good condition, is in the range of 96 – 100 per cent, supporting the fungus' success in infecting and causing insect death.

Incubation is placed in a dark room, adapted to the habitat of *C. maculatus*, which is generally found in warehouses. This condition did not have a negative impact on the growth and virulence of *S. lanosoniveum* CG888. The conidia of this fungus have quite a good tolerance for production in light or dark conditions [19]. Apart from that, there have been no reported negative impacts on *S. lanosoniveum* to date. Some research reported that it is actually useful [20]. Therefore, it can be stated that *S. lanosoniveum* is able to induce mortality in *C. maculatus* pests and is safe as a biological agent for warehouse pests.

On day 11 [21], insect mortality from each treatment reached 100%, and was greater number died than controls. The highest mortality was in the application treatment of 10^8 conidia/ml which reached 86.7%. While the lowest mortality at 10^6 conidia/ml was 70%. Fig. 2 shows that the LT50 of *S. lanosoniveum* CG888 at a conidia density of 10^8/ml on *C. maculatus* F mortality was around day 6 - 7. *Beauveria bassiana* takes around 4.07 days with the same conidia density to kill *C. maculatus* at LT_{50} [22]. This shows that even though it has a slightly longer day range than *B. bassiana*, *S. lanosoniveum* CG888 has almost the same ability to cause the death of *C. maculatus* at LT_{50}. The difference in period is thought to be due to the condition of the fungus [23], including less strong release of conidia and less rapid ability of conidia to germinate and penetrate the exoskeleton. Therefore, further tests are needed to highlight the superior entomopathogenicity of *S. lanosoniveum* CG888 to cause rapid death of *C. maculatus* F.

4 Conclusion

Simplicillium lanosoniveum CG888 is capable of causing the death of *Callosobruchus maculatus* F. at a density of 1x10^8 conidia/ml, reaching 51.65% compared to other treatments and control. Based on the length of time after inoculation, there were 60.83% *C. maculatus* F. deaths on the 11^{th} day. It was higher than the results on 3, 5, 7 and 9 days later. There was a positive interaction that the mortality of *C. maculatus* F was influenced by the density of *S. lanosoniveum* CG888 conidia and the period of days after inoculation. Among these treatments, *S. lanosoniveum* CG888 with 1x10 8 /ml caused a mortality of 86.7% in *C. maculatus* F. on day 11 after inoculation. The lowest mortality of *C. maculatus* F. was in the density of 1x10^6 conidia/ml of 70%. These findings indicate that *S. lanosoniveum* CG888 can be used to control *C. maculatus* F. safely and reduce the use of chemicals in soybean storage.

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