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## POTENTIAL ENTOMOPATHOGENIC FUNGI TO CONTROL SCALE INSECT PEST ON CITRUS TANGERINE (*CITRUS SUHUIENSIS* TAN.)

Triwiratno A.\*, Wuryantini S., Agustina D.

Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), Batu, Indonesia

\*E-mail: [anangtriwiratno@gmail.com](mailto:anangtriwiratno@gmail.com)

### ABSTRACT

Achieving of food self-sufficiency can be done by using of local potential that is by agribusiness in Indonesia. One potential locally owned citrus agribusiness was the use of entomopathogenic fungi to improve the productivity of citrus. Reports showed decrease in productivity due to infestation of scale insect. The experiment was conducted at the Integrated Laboratory of Indonesian Citrus and Subtropical Fruit Research Institute in October 2013 to October 2014. The study began with a survey for scale insect infestation on citrus crops in high land, medium land and low in dry and rainy seasons. Taken from a collection of entomopathogenic fungi associated with scale insect in the field. Collection of fungi isolated from single conidia and its ability to infect selected scale insect. Entomopathogenic fungi were further tested for the viability and pathogenicity against scale insect. The results showed that the scale insects attacked citrus were types of *L.beckii* and *A.Aurantii*. The highest attack occurred at low land during the dry season by *L.beckii* with population of 4.2 heads increased to 5.5 individuals per 10 cm in the rainy season. Viability test results showed that the isolates had viability above 50% were SKB4K, SKD1K and SBB3K for 73.6, 61.6 and 53% respectively, which were collected during the dry season. While isolates obtained in the rainy season were SBWD2H and SBWD3BH, each with viability of 77.3 and 78.3% respectively. Pathogenicity test results showed that there were 6 isolates known to have potential as entomopathogenic fungi for controlling scale insect, namely, SBWB2H, SBWD2H, SBWD3BH, SKD1K, SBWD1K and SBB3K which had pathogenicity over 50% up to 14 days.

### KEY WORDS

Citrus, scale insect, entomopathogen fungi, viability, pathogenicity.

Problem encountered in developing citrus as one of national fruits is the low fruit quality. The low productivity is partly due to the existence of pest infestation of scale insect. Scale insect is now a major pest of citrus, whose population is very high, and causes damage to the production of citrus fruits (Meekes, 2001, Meekes et al., 2002; Triwiratno, 2004; Triwiratno and Yunimar, 2005). Scale insect with high population density attacking citrus plants causes leaves and fruits fall before ripening, as well as twigs and stems of plants die (Triwiratno et al, 2003a).

Brown scale insect (*L. beckii*) have natural enemies from fungi member of family *Aschersonia* (Meekes, 2001; Meekes et al., 2002; Triwiratno, 2004; Triwiratno and Yunimar, 2005; Liu et al., 2005; Jun-Zhi et al., 2005; Dolinski and Lacey, 2007). The fungi can control brown scale insect because they produce secondary metabolites which is insecticidal, namely destruxins A4 and A5 which is a compound of depsipeptida (Krasnoff and Gibson, 1996) and Ascherxanthone A (Isaka et al, 2005).

Citrus crops in Indonesia are planted and cultivated commercially in lowland (0-400m above sea level (asl), medium land (400-800m above sea level), and highland (> 800m asl). Commercial citrus varieties most widely grown in Indonesia are tangerine (*C. suhuiensis* Tan.) with a population of 75%, mandarin (*C. reticulata*) with a population of 10%, and pummelo (*C. grandis*) with a population of 6% of all citrus population grown in Indonesia (Balitjestro, 2010).

This study aims to (1) determine the type and population of scale insect that attack citrus plants on three types of elevation during dry season and rainy season. (2) Selection, viability testing, and pathogenicity of fungal isolates against scale insect.

### **MATERIALS AND METHOD of research**

Research conducted at the Integrated Laboratory of Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), Tlekung, Batu. The research was conducted in the dry season and rainy between October 2013 and October 2014. Location for observation of scale insect population and sampling of fungi associated with scale insect at highland was Bangli Regency, Bali; at medium land was Banyuwangi Regency, East Java; and at lowland was Sambas Regency, West Kalimantan.

*Isolation of fungi associated with scale insect and propagate single conidia.* Technique for isolation of fungi associated with infestation of scale insect was performed per method of Liu et al. (2005 and 2006) and del Prado et al. (2008). Samples of scales section infested with the fungus was sampled using a needle loop and inoculated into petri dishes containing PDA (Potato Dextrose Agar) medium comprising of 50 mg / L teramisin. Fungal cultures incubated at a temperature of 25-30 ° C for approximately 7-14 days until fungal colonies filled the cup. Each colony of fungi that grow was subsequently rejuvenated into a PDA medium and incubated to produce conidia period.

*Rearing scale insect.* Propagation (rearing) of scale insect used Banjar tangerine as host plant, planted in polybags diameter of 30 cm. 20-30 heads of first instar larvae (crawlers) of brown scale insect still actively moving (aged 1 day) were taken from a citrus plant having severe infestation of brown scale insects and inoculated onto each green leaf. The edges of leaves inoculated with crawler were limited by using wet tissue paper folds to prevent the leaving of the larvae of the leaf. The larvae of brown scale insect were reared in a screen house at a temperature of about 25°C until imago reached 30 days and ready for treatment.

*Selection of entomopathogenic fungi.* Selection of entomopathogenic fungi was done against isolates that have the phenotype of entomopathogenic and have the ability of Lethal Concentration 50 (LC 50) within 14 days. Pure fungi were isolated on PDA then incubated at 25-30°C for ± 30 days or until colonies filled the petri dish. The number of conidia was calculated by Haemocytometer to achieve the density of 107 conidia / ml. Suspension was aseptically put into handsprayer using a micropipette. The percentage of mortality was calculated by formula (Wahyono and Tarin, 2007).

*Viability of entomopathogenic fungi conidia.* Results obtained from the selection that had the ability above LC 50 at 14 days, meaning that they were pathogenic against scale insect, continued counting the viability of the fungi. Fungal colonies from selection results were grown on PDA to fulfill the cup. Conidia were harvested by adding 10 ml of sterile distilled water containing 0.02% Tween 80 into the cup to form a suspension containing cultured conidia masses. The number of conidia on mass suspension was counted with Haemocytometer until reaching density of 102 conidia / ml by serial dilution then 0.1 ml was taken using a micropipette and spread onto PDA medium surface in petri dishes by using spread plate method and flattened with dry glassy (Alves et al., 1998 cit. Francisco, 2006). Conidia in PDA medium incubated at room temperature for 24 hours to form conidia germination (Skrobek, 2001).

*Fungus pathogenicity test against scale insect.* Pathogenicity test was merely done to isolate of selection results. The experimental design used in the study was Random Block Design factorial with three variables. The first variable was fungal isolates; the second variable was density of application conidia; and the third variable was days of observation. Replications were three times. Total conidia calculated by Haemocytometer. Dilution was to obtain conidia density of 102-107 conidia / ml. Suspension was aseptically put into handsprayer using a micropipette. The percentage mortality was calculated by formula (Wahyono and Tarin, 2007).

**RESULTS AND DISCUSSION**

*Survey result of scale insect population on tangerine.* Scale insect that attacked citrus plants was a type of *L. beckii* and *A. Aurantii*. The highest attack occurred on the stems of citrus grown in the lowlands with the scale insect population of *L. beckii* was 4.2 heads per 10 cm rod (Figure 2). High scale insect populations in the lowland supposedly linked to conditions of high humidity on the tangerine plant, where the sample collected in the district of Sambas, West Kalimantan generally known of having rainfall throughout the year for 12 months.

*L. beckii* liked the dense tree canopy, and severe attacks usually occur in the central part of the tree canopy (Futch et al., 2001; Knapp, 2003; Triwiratno, 2004; Anonymous, 2007). The attacks on the stems, leaves and fruits found on the plant in the field caused typical symptoms of damage on the surface and the appearance of dotted and dull.

Imago *L. beckii* is dark brown with varied shapes that was long, circular and coma. Scale insect generally has a size of 1.0 to 3.0 mm and has a sort of shield on his back. Scale insect reproduce sexually or parthenogenesis. Most female scale insects can produce 40-80 eggs and placed in groups around the body that will hatch on the eighth day after the egg is produced. In the dry season, the eggs hatch in 15-20 days, while in the rainy season, hatching time is longer (Fasulo and Brooks, 2004). Crawler of scale insect was white and runs very slowly, usually found on the stems and leaves sidelines. Crawler can survive for three days without food nutrients and can move only a few hours, then settled on a part to develop into adults (Grafton et al., 2000).

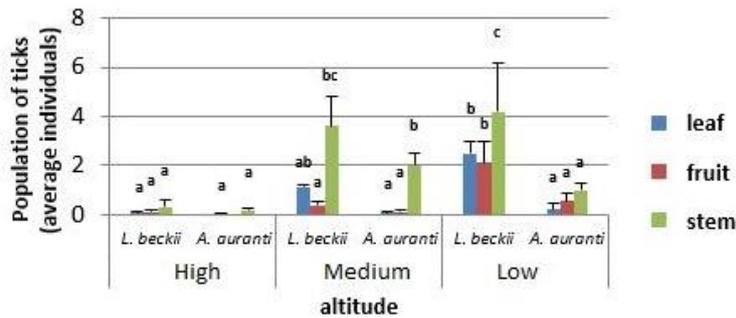


Figure 1 – Population and species of scale insects that attacked tangerine (*C. suhuiensis* Tan.) at highland, medium land and low land in dry season

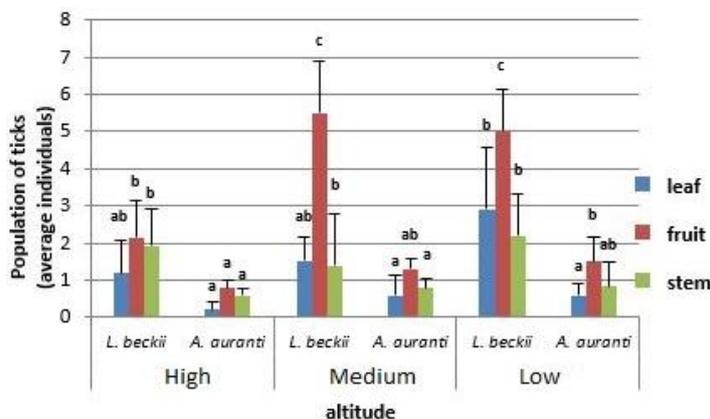


Figure 2 – Population and species of scale insects that attacked tangerine (*C. suhuiensis* Tan.) at high land, medium land and low land in rainy season.

Female insect changed skin twice, while the male had four skin changes before they reached the adult stage with wings. In a year, usually there are three or more generations. Scale insect can survive on host plants by sucking fluids from the leaves, fruit, branches, and

stems of its host plant, causing chlorosis, leaf drop, incompleting ripening, abscission of fruits, dried branches and plant death (Fasulo & Brooks, 2004).

Observations of the attacks carried out in rainy season was in conjunction with the fruit began to grow. The existence of new growing fruits stimulated transfer of the crawler to move to get a young plant parts that will be used as a place to live when he became imago. The highest scale insect population was *L. beckii* at the lowlands and the medium that was between 5 to 5.5 heads per fruit (Figure 2). While the population on the stems and leaves was lower.

*A. Aurantii* female mite had a hard body covering, round-shape and was maroon. While the body covering of the male larvae was oval and smaller. Body shield of the of the female adult had a diameter between 1.5-2 mm. The larvae were brown with a very small body size (Amitaningsih, 2005). According Efendi (2009) Adult female was oval, had diameter of 2 to 2.3 mm, was spherical orange or dark brown, and produce 60-150 crawlers (first instar larvae were active).

*Selection of entomopathogenic fungi from host plant type oftangerine (Citrus suhuiensis Tan.) in dry season and rainy season.* Isolated fungi from three altitudes resulted 12 fungus isolates suspected entomopathogenic against brown scale insect, i.e SB B1 K, SB B2 K, SB B3 K, SB D1 K, SB D2 K (from Bali), SBW D1 K, SBW D2 K, SBW D3 K, SBW B1 K (from Banyuwangi), KSB4, KS D1 K, KS D2 K (from West Kalimantan). Fungus samples inoculated on PDA mostly obtained from the leaves and stems infected by brown scale insects. According Wraight et al, (2007) entomopathogenic fungi are adapted to dry conditions with sufficient moisture to actively infect pests (for example, on the abaksial surface of leaf or in the fold of insect cuticle membranes). Generally, entomopathogenic fungus infection against brown scales marked by orange or yellow fungal hyphae attached around the body of the insect.

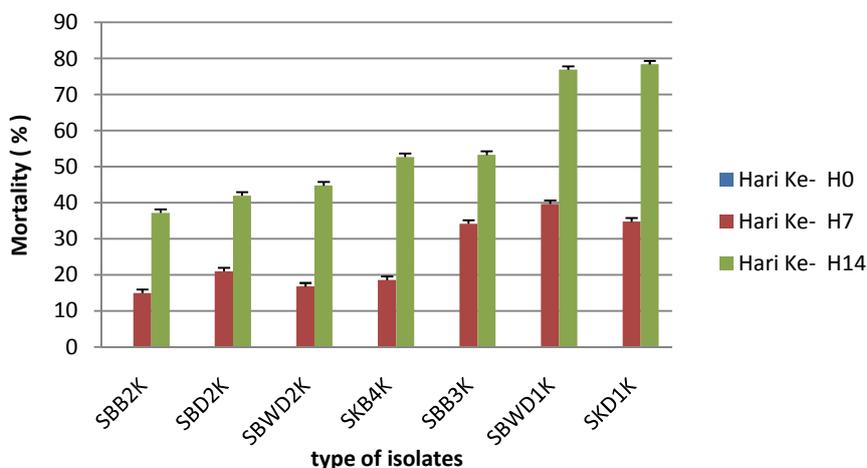


Figure 3 – Mortality of *L. beckii* by seven isolates of entomopathogenic fungi isolated from scale insect on tangerine host plant in dry season

Mortality data from the selection trials resulted four species isolates that have the highest pathogenicity, namely, SK D1 K (Sambas), SBW D1 K (Banyuwangi), SB B3 K (Bali) and SK B4 K (Sambas) with a mortality rate of *L. beckii* was 78.4%, 76.9%, 53.3% and 52.7% respectively in 14 days. Other isolates were only able to control *L. beckii* less than 50% for 14 days (Figure 3). Isolates obtained from the same host insect but of different topographic had different virulence (Fatiha et al, 2007). This was shown from *L. beckii* mortality data produced by seven different isolates. SK D1 K isolates originating from the lowlands had the highest degree of pathogenicity compared to other isolates. This was because the population of host plant in the lowland was better. In addition, the environmental conditions at the low land could create the characters of the physiology of fungi that were more virulent than the others originating from the highlands and medium.

Isolated fungi from three altitudes resulted nine isolates of the fungus suspected entomopathogenic against brown scale insect, i.e. SB B1 H, SB B2 H, SB D2 H, SB D3 H (from Bali), SBW D2 H, SBW B2 H, SBW H D3B, D3C SBW H (from Banyuwangi), and SK B1 H (from West Kalimantan) (Figure 4).

Alavo et al, (2004) asserts that the host range and ecological conditions can influence the genetic diversity that directly affect the virulence of a fungus power. Zhen et al, (2005) states that the virulence of entomopathogenic fungi is influenced by the character of physiology. Meanwhile, physiology character of entomopathogenic fungus closely related to the rate of growth, sporulation, conidia germination and tolerance to temperature differences.

According Prayogo (2006) the concentration of fungi with conidia density of 107 conidia / ml is the standard concentration in testing biological products. Prayogo & Marwoto (2005) also declare that the minimum dose of conidia fungal pathogens that can lead to death of insects is 103 conidia / ml. Previous research has shown that *B. bassiana* entomopathogenic fungi on the density of 107 conidia / ml could infect termites up to 100% (Desyanti et al., 2005).

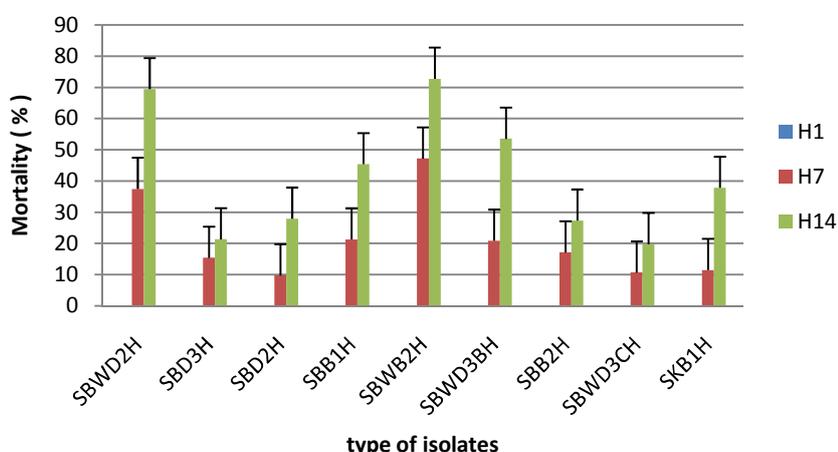


Figure 4 – Mortality of *L. beckii* by nine isolates of entomopathogenic fungi isolated from scale insect on tangerine host plant in rainy season

*Viability of selection result of entomopathogenic fungi in dry season and rainy season.* A total of seven isolates of the selection result, namely, SB B2 K, SB B3 K, BSD2, SBW D1 K, SBW D2 K, SK B4 K and SK D1 K (Figure 5) were tested for conidia viability to identify the speed of conidia germinated within 24 hours. The ability of fungi conidia to germinate within a certain time could be seen from the level of its viability. The higher the percentage of viability, the shorter the germination time required.

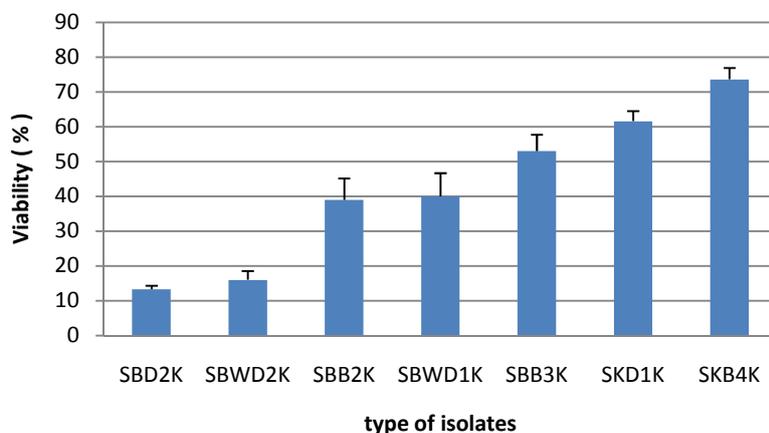


Figure 5 – Viability of conidia of seven entomopathogenic fungi isolated from scale insect on tangerine host plant in dry season

Different isolates types had a significant influence on the value of the viability of conidia of the fungal isolates ( $P < 0.05$ ). Of the seven tested isolates, there were three types of isolates that had the highest level of viability of fungal isolates conidia, namely, SK B4 K, SK D1 K and SB B3 K of 73.6%, 61.6% and 53% respectively, while four other isolates had a percentage viability of less than 50% (Figure 5). Viability of isolates conidia of SB B3 K was relatively lower than isolates SK D1 K, even though the two isolates were statistically not significantly different ( $P > 0.05$ ). Based on the results of previous studies, the percentage of fungal conidia viability could reach 90% - 100% (Rahayu, 2009). The highest viability was achieved by SBW D2 H and SBW D3B H with viability of 77.3% and 78.3% respectively (Figure 6). In this viability test, fungal isolates were incubated at room temperature of 25 ° C. The optimum temperature for growth, pathogenicity and survival of entomopathogenic fungus was around 20 - 30°C (Morissey & Osbourn, 1999).

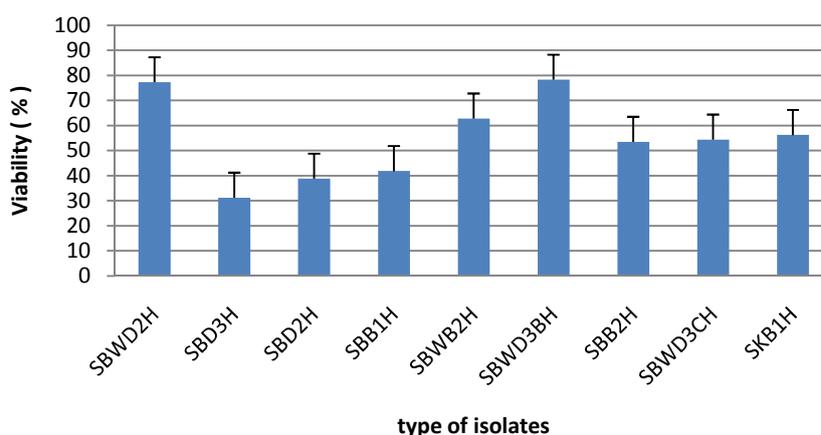


Figure 6 – Viability of conidia of nine entomopathogenic fungi isolated from scale insect on tangerine host plant in rainy season

Viability of entomopathogenic fungus spore was influenced by temperature, humidity, pH, solar radiation and chemicals, such as nutrients and pesticides (Muller Kongler, 1967 in the Robert & Yendol, 1971; Riyanto, 1993). In this viability test, fungal isolates were incubated at room temperature of 25°C. The optimum temperature for growth, pathogenicity and survival of entomopathogenic fungus was around 20 - 30°C (Morissey & Osbourn, 1999).

*Pathogenicity of entomopathogenic fungi from tangerine host plants.* A total of six isolates were tested for pathogenicity to determine the level of virulence. All three isolates were selected based on the selection test with mortality, and the highest conidial viability were SBW D1 K, SK D1 K, SB B3 K, SBW D2 H, SBW B2 H, and SBW D3 BH with mortality of *L. beckii* was 81.1%, 73.6%, 68.8%, 69%, 72%, and 54% respectively, for 14 days at a concentration of 107 conidia / ml. Observation of the first day of treatment showed that all concentration treatment of each type of isolates had not shown *L. beckii* death.

The increase of *L. beckii* deaths can be observed on the seventh day and 14th day after the application. Increased mortality rates can be compared with the control treatment. In this case, the control treatment merely contained tween 80. Of all the control treatment, the mortality rate of each isolate was 0%. Differences in density of fungus conidia had a significant influence on mortality of *L. beckii* of each isolate type ( $P < 0.05$ ).

Isolates of SB B3 K generated from the highlands of Kintamani-Bali had conidial viability rate of 53% on PDA. Although the percentage of the conidia viability was medium, this isolates at selection test could control *L. beckii* up to 53.3% at a concentration of 107 conidia / ml for 14 days. Based on this result, the SB B3 K isolates was further tested to determine the pathogenicity against *L. beckii*.

Significant difference between conidia density and observation time indicated an interaction between them ( $P < 0.05$ ). Differences in conidia density gave effect to the increase in the value of the percentage of brown scale insect mortality. Isolates of SB B3 K could

control *L. beckii* at the highest concentrations of  $10^4$ ,  $10^7$  and  $10^3$  conidia / ml for 70.8%, 68.8% and 59.9% respectively at day 14<sup>th</sup>. Isolates of SB B3 K at the concentration of  $10^4$  conidia / ml could control *L. beckii* higher than that at the concentration of  $10^7$  conidia / ml, as well as that at the concentrations of  $10^5$  and  $10^6$  conidia / ml could control *L. beckii* lower than that at the concentrations of  $10^4$  and  $10^3$  conidia / ml (Figure 7).

This study indicated that a high conidia density did not always give a high mortality rate. This condition is contrary to previous statement that the higher conidia given, the higher mortality generated. This was caused by the host population density and environmental conditions. LC50 value generated from this isolates SB B3 K was  $4,5 \times 10^7$  conidia / ml on day 14<sup>th</sup>.

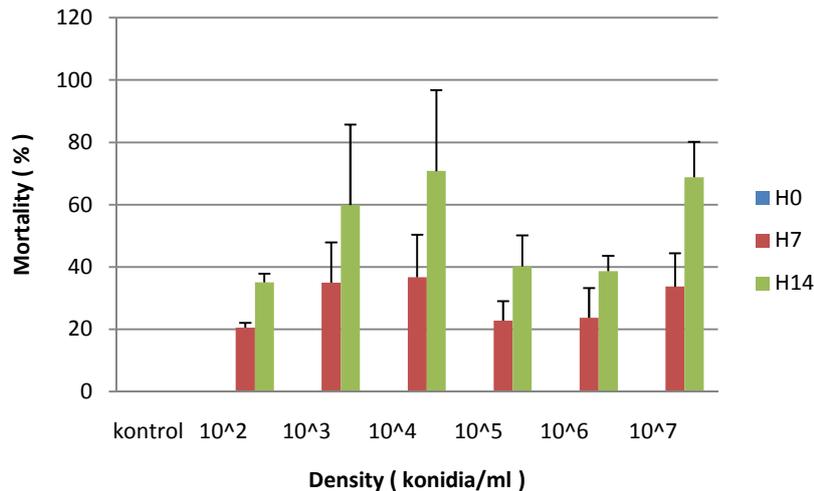


Figure 7 – Mortality of *L. beckii* by isolates of SB B3 K with density of  $10^2$ - $10^7$ /ml at 1<sup>st</sup> day, 7<sup>th</sup> day and 14<sup>th</sup> day

Isolates of SBW D1 K came from the medium land in Banyuwangi had a viability level of 40% which were grown on PDA. Although the percentage of viability was relatively very low (<50%), this isolates, at the selection phase, could control *L. beckii* up to 76.9% at a concentration of  $10^7$  conidia / ml for 14 days. Isolates of SBW D1 K was suspected can germinate well when having direct contact with the host than on PDA medium containing high carbohydrates. According to Nelson et al, (1983), high carbohydrate content causes a loss of viability of entomopathogenic fungi. Based on this, the isolates of SBW K D1 was further tested to determine the ability of pathogenicity against *L. beckii*.

Isolates of SBW D1 K could control *L. beckii* mostly at the concentrations of  $10^7$ ,  $10^6$  and  $10^5$  conidia / ml for 46.2%, 42.4% and 35.4% respectively on the seventh day. Mortality value on the seventh day was different from the fourteenth day. The longer the time the application made, the higher the value of *L. beckii* mortality resulted from each concentration. On day fourteenth, mortality at concentrations of  $10^7$ ,  $10^6$  and  $10^5$  conidia / ml increased by 81.13%, 63.5% and 51.2% respectively, whereas at lower concentrations, that were,  $10^4$ ,  $10^3$  and  $10^2$  conidia / ml could control *L. beckii* less than 50%, i.e. 48.1%, 38.7% and 25% on the fourteenth day. The LC50 value generated from isolates of SBW D1 K was  $3,3 \times 10^6$  conidia / ml.

Isolates of SK D1 K came from the lowland in Sambas, West Kalimantan. These isolates had conidial viability value of 61.6% on PDA medium. The results of selection test indicated that the mortality rate of *L. beckii* reached 78.4% at day 14<sup>th</sup>. The ability of these isolates to infect *L. beckii* was high as well as its viability rate, therefore, it was necessary to test the isolate pathogenicity.

On the seventh day, the isolates of SKD1 K could control *L. beckii* at concentration of  $10^5$ ,  $10^6$  and  $10^7$  conidia / ml for 25.8%, 23.5%, and 20.4% respectively. The value of mortality increased up to the fourteenth day. On day 14<sup>th</sup>, the mortality of *L. beckii* at concentration of  $10^7$  and  $10^6$  conidia / ml increased more rapidly than that at the concentration of  $10^5$  conidia /

ml for 73.6%, 69, 9% and 56% respectively. Whereas the isolate at concentration below  $10^4$ ,  $10^3$  and  $10^2$  conidia / ml could control *L. beckii* less than 50%, i.e. 45.4%, 32.4%, and 28% respectively at day 14<sup>th</sup>. The LC50 value generated from isolates of SK D1 K was  $3,3 \times 10^5$  conidia / ml.

Isolates of SBW D2 H collected from host plants at the medium land in Banyuwangi, East Java were isolated from leaves during the rainy season. Based on the LC50, isolates of SBW D2 H were the most virulent to *L. beckii* since the conidia of  $10^3$  conidia / ml on day 14<sup>th</sup> could infect 50% of the scale insect. The LC50 value from isolates of SBW D2 H was  $7,2 \times 10^6$  conidia / ml.

Isolates of SBW B2 H collected from host plants at the medium land in Banyuwangi, East Java were isolated from leaves during the rainy season. Based on the LC50, isolates of SBW D2 H were the most virulent to *L. beckii* since the conidia of  $10^3$  conidia / ml on day 14<sup>th</sup> could infect 50% of the scale insect. The LC50 value from isolates of SBW B2 H was  $1,04 \times 10^6$  conidia / ml.

Isolates of SBW D3 BH collected from host plants at the medium land in Banyuwangi, East Java were isolated from leaves during the rainy season. Based on LC50, isolates of SBW D3 BH had a lower virulence than isolates of SBW B2 H, because the conidia of  $10^5$  conidia / ml on day 7<sup>th</sup> could infect only 50% of the scale insects, whereas isolates of SBW B2 H could get LC50 at conidia of  $10^4$  conidia / ml. The LC50 value from isolates of SBW D3 BH was  $5.3 \times 10^4$  conidia / ml.

## CONCLUSION

Scale insects that attacked tangerine (*C. Suhuiensis* Tan.) at the highlands, medium lands, and low lands during the dry season and the rainy season were types of *L. beckii* and *A. Aurantii*. The highest population occurred at the low lands during the dry season by *L. beckii* with a population of 4.2 heads and increased to 5.5 heads per 10 cm in the rainy season. Selection result, viability test and pathogenicity showed that there were six fungal isolates that have potential as entomopathogenic fungi to control scale insects, namely, SBW B2 H, SBW D2 H, SBW D3 BH, SK D1 K, SBW D1 K and SB B3 K.

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