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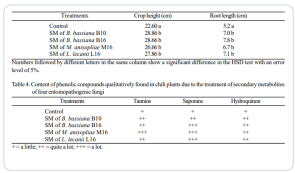

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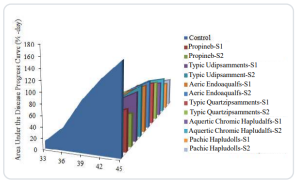

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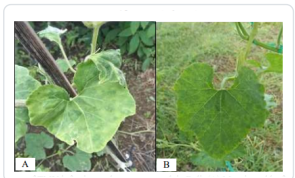

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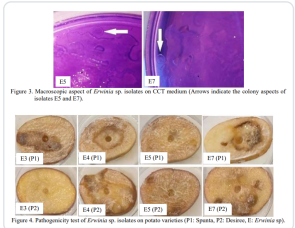

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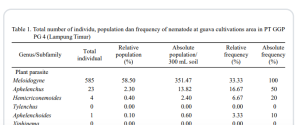

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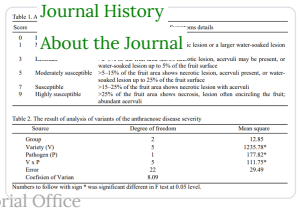

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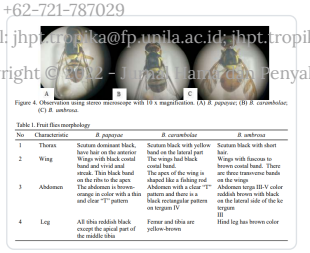

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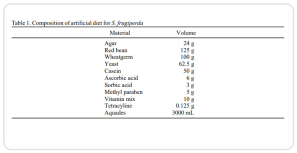

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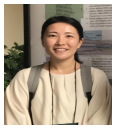

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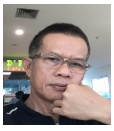

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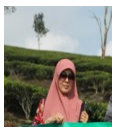

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CROSS APPLICATION OF ENTOMOPATHOGENIC FUNGI RAW SECONDARY METABOLITES FOR CONTROLLING FUSARIUM WILT OF CHILI SEEDLINGS

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ABSTRACT

Cross application of entomopathogenic fungi raw secondary metabolites for controlling fusarium wilt of chili seedlings. The research aimed to determine the effect of entomopathogenic fungi raw secondary metabolites on fusarium wilt on chili plants and on growth of chili. In vitro test used a Completely Randomized Design with 5 treatments and 5 replicate and in planta using a Randomized Block Design with 5 treatments and 5 replicatie including control, secondary metabolites of *Beauveria bassiana* B10, *B. bassiana* B16, *Metarhizium anisopliae* M16, dan *Lecanicillium lecanii* L16. Variables observed included inhibition ability, incubation period, desease intensity, plant height, root length, and phenolic compounds (tannins, saponin, and hydroquinone) content qualitatively. The results showed that secondary metabolites of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 were able to inhibit growth of *Fusarium oxysporum* f.sp. *capsici* by 50.62; 50,64; 48,62; 56.62%, respectively, extend incubation periods of 71.05; 73,38; 64.89; and 68.57%, respectively, suppress disease intensity by 99.99; 99.99; 99.99; and 99.99%, respectively, can increase plant height by 15.22; 18.8; 21.14; 21.69%, respectively, increasing the root length by 22.61; 25,71; 26,34; 33.50%, respectively, and can increase the content of tannins, saponins and hydroquinone compounds qualitatively compared to controls. The secondary metabolites of enthomopathogenic fungi could be used as organic control for soilborne pathogenic fungi.

Key words: chili plants, fusarium wilt, entomopathogenic fungi, secondary metabolites

INTRODUCTION

Chili pepper (*Capsicum annum* L.) was an important horticultural crops in the tropics and subtropics (Baenas *et al.,* 2018; Olatunji & Afolayan, 2018). Cultivation of chili crops was inseparable from pest disturbances. One of the disorders that occur in chili crops was wilt disease caused by *Fusarium oxysporum* f.sp. *capsici* (Gabrekiristos & Demiyo, 2020). *F. oxysporum* f.sp. *capsici* caused a high loss of chili production (Velarde-Félix *et al.,* 2018; Gabrekiristos & Demiyo, 2020) and was able to survive in the soil for a long time as chlamydospores even though there were no host (Gordon, 2017; Altinok *et al.,* 2019). Transmission could occur through soil and planting material derived from diseased plants and could infect host plants through wounds on the roots (Velarde-Félix *et al.,* 2018).

So far, fusarium wilt control still depended on fungicides (Bashir *et al.,* 2018). However, with the increasing awareness of consumers and the negative impacts of fungicide, including the emergence of a new fungicide-resistant strain of Fusarium, it was necessary to use other alternative controls that were environmentally friendly and safe (Besset-Manzoni *et al*., 2019). One of the controls that could be carried out to control fusarium wilt in chili plants was by using biological agents.

Several biological agents have been tried, but the results was fluctuate, this due to *F. oxysporum* as a soil-borne fungus that difficult to control (Köhl *et al*., 2019). Entomopathogenic fungi were biological agents that have been used to control insect pests and were generally spore-based (Mora *et al*., 2017; Litwin *et al.,* 2020). The application of spore-based biological agents in the field encountered several obstacles, including (a) stress of abiotic factors, such as temperature, humidity, and sunlight which would affect conidium germination and spore production (Hsia *et al*., 2014; Velivelli *et al*., 2014), (b) propagation medium, had an effect on the stability of conidium and blastopore production, reduced sources of protein, carbon, starch, and chitin in the propagation medium could decrease the quality of the entomopathogenic fungi spores, so that it needed to be overcome by using secondary metabolites (Roshandel *et al*., 2016; Corrêa *et al*., 2020).

Secondary metabolites are inherent genetic properties of an organism. Several entomopathogenic fungi are known to produce secondary metabolites that were

biologically active (Fernandes *et al*., 2012; Kim *et al*., 2013; Gustianingtyas *et al.,* 2020). Secondary metabolites of entomopathogenic fungi contain various compounds, especially the chitinase (Fernandes *et al*., 2012; de Laguna *et al*., 2015; Altinok *et al*., 2019), which degrade chitin, and which also make up the conidium layer of pathogenic fungi. *Beauveria bassiana* had shown antifungal activity against *F. oxysporum*, *F. oxysporum* f.sp. *cepae*, *F. oxysporum* f.sp. *lycopersici*, *Armillaria mellea*, *Rosellinia necatrix*, *Botrytis cinerea*, *Pythium ultimum*, *P. debaryanum*, *P. myriotylum*, *Septoria nodorum*, and *Rhizoctonia solani* (Ownley *et al*., 2010). According to Jaber & Ownley (2018), the possible mechanisms of protection conferred by endophytic fungal entomopathogens were as dual microbial control agents against both insect and pathogen pests. Based on these secondary metabolites, it is necessary to try cross-application of the secondary metabolites of entomopathogenic fungi to plant diseases. This study aimed to determine the effect of entomopathogenic fungi secondary metabolites on fusarium wilt disease in chili plants and on the growth of chili plants.

MATERIALS AND METHODS

Research Site. The research was carried out in the screen house for 4 months and the preparation was done in the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto.

Preparation of Enthomopatogenic Fungi. Each of the entomopathogenic fungi *B. bassiana* B10, *B. bassiana* B16, *Metarhizium anisopliae* M16, and *Lecanicillium lecanii* L16 (Loekas Soesanto collection) were purified on Potato Dextrose Agar (PDA) media (Fitriana *et al*., 2018). Each culture was incubated at room temperature for 7 days.

Preparation of *F. oxysporum* f.sp. *capsici*. Preparation of the plant pathogenic fungi *F. oxysporum* f.sp. *capsici* was performed by isolating chili plants with the symptom of fusarium wilt. Isolation was carried out by cutting the base of the infected chili plant, then sterilized with 70% alcohol by soaking for 1 min, after that rinsed with distilled water for 1 min. Furthermore, grown on PDA media and incubated at room temperature for 5 days (Kalman *et al*., 2020).

Preparation of Secondary Metabolites. As much as 20 g of rice flour and 10 g of granulated sugar were boiled with 1000 mL of water, then put into 10 glass bottles (100 mL, v/v). The mixture was sterilized using an autoclave at a temperature of 121 ^{o}C, a pressure of 15 psi for 30 min. After the mixture was cooled, each pure culture of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16, was taken from the PDA medium with a cork drill (10 mm in diameter) and each of 5 cork drills was put into the mixture and shaken with a shaker at 150 rpm at room temperature for 10 days (Soesanto *et al*., 2019).

Preparation of Chili Seedlings. Chili seeds were soaked for 1 hours according to treatment. The seeds were spread on a box (20 × 30 × 8 cm) filled with fine soils for 3 weeks then replanted in polybags according to the treatment. The planting medium used was unsterlized soil mixed with manure in a ratio of 2 : 1, then put in a 40 × 40 cm polybag.

In vitro Antagonism Test. In vitro antagonism tests were carried out between secondary metabolites of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16, and the phytopathogenic fungi *F. oxysporum* f.sp. *capsici*. The 7 days old culture of phytopathogenic fungi was taken using a cork drill (9 mm in diameter) then transferred to the new PDA in a petri dish using a spatula aseptically at a distance of 3 cm from the edge of the Petri dish. A Sterile filter paper (5 mm in diameter) was dipped in each of the entomopathogenic fungi secondary metabolites then placed aseptically to the same petri dish with a distance of 3 cm from the fungus (Živkovic *et al.,* 2010). The cultures then incubated at room temperature using a completely randomized design (CRD) with 5 treatments and 5 repeatation.

Application of Chili Seedlings. Planting holes were made for polybags containing planting medium, then inoculated the fungus *F. oxysporum* f.sp. *capsici* into the planting hole as many as 5 cork drill molds. After 5 days of transplanting, the secondary metabolites of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 were poured each of 50 mL/plant and repeated with the same volume of watering 10 days after transplanting. On the 15th and 20th days after transplanting the plants were watered with 100 mL/plant of entomopathogenic fungal secondary metabolites. The test used a randomized block design with 5 treatments repeated 5 times.

Inhibition Ability Test. Pathogen growth was measured and data on the inhibition of mycelia growth was calculated using a formula (Bekker *et al*., 2006):

$$P=\left(\frac{r_2-r_1}{r_2}\right)\times 100\%$$

P = percentage of inhibition (%);
r_1 = colony radius of *F. oxysporum* f.sp. *capsici* facing the fungal colony;
r_2 = radius facing the edge of the Petri dish

Incubation Period. The incubation period was calculated from the first day of inoculation of the pathogen until the first symptoms of disease appear in plants, with units of days after inoculation.

Disease Intensity. Disease intensity observations were carried out every week, since the first symptoms appeared, using the following formula (Abdel-Monaim & Ismail, 2010):

$$DI = \frac{\sum(v \times n)}{Z \times N} \times 100\%$$

DI = disease intensity (%);
V = infection category score;
N = number of plants attacked in each category;
Z = the hightes attack category score;
N = number of plants observed, with attack category: 0 = healthy% plants, 1 = wilted plants 1–20%; starting at the lower leaves and the base of the brown stems; 2 = 21–40% wilted plants, brownish rot at the base of the stems; 3 = 41–60% wilted plants, rotting the base of the stem is expanding but still on the surface of the soil; 4 = the plant is wilted 61–80%, the rot of the base of the stem is more than 5 cm and has reached the bottom; 8 = the plants is wilted > 81% and has reached the generative part.

Qualitative Content of Tannin, Saponin, and Hydroquinone Compounds. The phenol compound analysis was carried out at the end of the study qualitatively on the chili plant tissue. Tests carried out based on Rahmania *et al*. (2018) include tannin and saponin. Tannin, saponin, and hydroquinone tests were carried out by extracting 10 g of plant material with 80% ethanol then filtered and added 10 mL of distilled water. A total of 5 mL of plant extract was then put into a test tube. Three drops of $FeCl_3$ then added to the extract. Hydrolyzed tannins gave a blackish blue color, while tannin condensation gave a blue green color, then compared to the control (Bele *et al.,* 2010). Saponin test was carried out by taking 1 drop of lerak and then adding 10 mL of water (as a control) to the test tube. The filtered extract was put into a 5–10 mL test tube, then shaken vigorously for 30 sec and let stand for 30 min. The foam that was formed more than 3 cm from the surface of the solution means that it was positive for saponins. If the foam is formed a little, then add a little Na_2CO_3 solution (Ribeiro *et al.*, 2013). The foam condition that remains stable and hard indicates the presence of free fatty acids (Vidal *et al.,* 2018). The hydroquinone test was carried out based on Gull *et al*. (2016), which is modified. A total of 5 mL of extracted results added 5 drops of 10% NaOH. The red color indicates hydroquinone.

Growth Components. Variable of pepper seedlings growth components was crop height and root length.

Data Analysis. Data diversity was analyzed using the F test with an error rate of 5%. If significantly different, the HSD (Honest Significantly Difference) was carried out at an error level of 5%.

RESULTS AND DISCUSSION

Inhibition Ability Test. The growth of *F. oxysporum* f.sp. *capsici* was inhibited by secondary metabolites of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16, respectively 50.62; 50.64; 48.62; and 56.62% compared to control (Table 1). This was presumably because in the entomopathogenic fungi secondary metabolites tested contained compounds that were detrimental to the fungus *F. oxysporum* f.sp. *capsici* so that the growth of the pathogenic fungi is inhibited. This was in accordance with Gustianingtyas *et al*. (2020) that the compounds contained in the secondary metabolites of entomopathogenic fungi were in the form of extracellular

Tabel 1. Inhibition of growth of *F. oxysporum* f.sp. *capsici* by secondary metabolites of four entomopathogenic fungi on the 5th day of testing

Treatments	Growth inhibition (%)
Control	0 a
SM of *B. bassiana* B10	50.62 b
SM of *B. bassiana* B16	50.64 b
SM of *M. anisopliae* M16	48.62 b
SM of *L. lecanii* L16	56.62 b

Numbers followed by different letters show a significant difference in the HSD test with an error level of 5%.

enzymes, such as chitinase. Chitinase had a mechanism to degrade chitin, which is a constituent of the fungal conidia walls of *F. oxysporum* f.sp. *capsici* (Kumar *et al*., 2018). The enzyme content for each entomopathogenic fungus was different. This was shown by the inhibition of the enzyme on the growth of *F. oxysporum* f.sp. *capsici* versus control. *B. bassiana* fungus produced secondary metabolites, as did the other entomopathogenic fungi that were tested (Keswani *et al*., 2013).

Morphological observations on the hyphal structure of *F. oxysporum* f.sp. *capsici* against fungal entomopathogenic secondary metabolites showed structural change. The swelling of the pathogenic fungal hyphae was thought to be due to lysis activity due to cell wall lysis enzymes, which contained in the secondary metabolites of entomopathogenic fungi (Molnar *et al*., 2010). Petrisor & Stoian (2017) stated that secondary metabolite compounds that enter fungal cells would cause mycolysis. Mycolysis was the loss of protoplasm in the cell wall structure so that the enzyme does not dissolve in the fungal cell wall. Mycolysis could cause thickening, shortening, and lysis of the walls so that the growth of hyphae becomes abnormal.

Effect of Secondary Metabolites of Four Entomopathogenic Fungi on Pathosystem Components. The incubation period of *F. oxysporum* f.sp. *capsici* (Table 2) showed that the treatment of entomopathogenic fungi secondary metabolites had a significant effect when compared to the control. The entomopathogenic fungal secondary metabolites could all prolong the incubation period. This was presumably because the control plants were not treated with entomopathogenic fungal secondary metabolites, so the plants did not had resistance to infection of *F. oxysporum* f.sp. *capsici*. This was in accordance with the opinion of Leclerc *et al.* (2014) stated that the shorter incubation period indicates a high degree of host pathogen suitability.

The application of secondary metabolites from *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 was able to prolong the incubation period (Table 2). It was assumed that the application of entomopathogenic fungi secondary metabolites could inhibit the growth of *F. oxysporum* f.sp. *capsici* due to the presence of annoying toxic compounds. As reported, the entomopathogenic fungi produce secondary metabolites, which cause the development of pathogens to be inhibited, thus affecting the incubation period (Molnar *et al*., 2010).

In line with the incubation period, four secondary metabolites of entomopathogenic fungi emphasized disease intensity. Based on Table 2, the entomopathogenic fungal secondary metabolites was significantly different when compared to controls. Even the inter-treatment of entomopathogenic fungal secondary metabolites had the same effect, and was able to suppress the intensity of the disease (Figure 1). It is suspected that the active compound present in the secondary metabolites of entomopathogenic fungi are able to inhibit the infection of the pathogen *F. oxysporum* f.sp. *capsici*.

This was in accordance with the opinion of Błaszczyk *et al.* (2021), that *L. lecanii* produces toxic secondary metabolites, namely bassionolidae and dipicolinic acid. *L. lecanii* and other enthomopathogenic fungi secretes a small amount of α-1,3 gluconase and protease enzymes which function to degrade cell walls (Mondal *et al*., 2016). The ability of enthomopathogenic fungi to control plant diseases was proven by Rustiguel *et al.* (2012). Kim *et al*. (2013) and Litwin *et al*. (2020) that entomopathogenic fungi secondary metabolites acted as pesticides by extracelular enzymes.

In addition, the application of entomopathogenic fungal secondary metabolites was able to increase plant defence against *F. oxysporum* f.sp. *capsici*. This was supported by a qualitative analysis of the plant phenolic compounds content (Table 4). Phenolic compounds were parameters of biochemical impacted plant resistance, which can overcome pathogen attack. The increase in plant phenolic content was due to the presence of foreign compounds that enter the plant tissue, in this case the secondary metabolites of entomopathogenic fungi that were applied (Litwin *et al*., 2020). Plants that were

Table 2. Incubation period and intensity of fusarium wilt disease in secondary metabolites treatment of four entomopathogenic fungi

Treatments	Incubation period (dai)	Disease intensity (%)
Control	6.6 a	21.33 a
SM of *B. bassiana* B10	22.8 b	0.01 b
SM of *B. bassiana* B16	24.8 b	0.01 b
SM of *M. anisopliae* M16	18.8 b	0.01 b
SM of *L. lecanii* L16	21.0 b	0.01 b

Numbers followed by different letters in the same column show a significant difference in the HSD test with an error level of 5%.

systemically resistant and contain compounds in the secondary metabolites of entomopathogenic fungi would be able to overcome the attack of pathogenic fungi, so that the disease intensity decreases (Sharma & Gupta, 2020). The active ingredients in the entomopathogenic fungal secondary metabolites enter the plant tissue through root absorption. Furthermore, secondary metabolite compounds were transported throughout the plant tissue (Barra-Bucarei *et al*., 2020).

Effect of Secondary Metabolites of Four Entomopathogenic Fungi on Growth Components. The treatment of each secondary metabolite of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 showed significant differences in plant height or able to increase plant height (Table 3). In the control, the lowest plant height was thought to be disturbed by nutrient absorption from the soil, because the roots of the plants were broken due to *F. oxysporum* f.sp. *capsici* infection. According to Bani *et al.* (2018), *Fusarium* sp. infection was spread from the root to the entire plant through the xylem vessels, thus interfering with the process of water transport and absorption of nutrients in plants and eventually the plant withers. When attacking chili plants, *Fusarium* sp. cause the roots to form a pile or colony at the base of the plant stems, the fungus would take the nutrients the plant needs, as a result, the food supply to the roots that should be distributed to plant tissue was reduced (Farahani-Kofoet *et al.,* 2020).

Figure 1. Application of entomopathogenic fungal secondary metabolites on fusarium wilt of chili seedlings. (A) Control; (B) Treated chili seedling.

Table 3. Differences in average plant height and root length of chili crops in the treatment of secondary metabolites of four entomopathogenic fungi

Treatments	Crop height (cm)	Root length (cm)
Control	22.60 a	5.2 a
SM of *B. bassiana* B10	28.86 b	7.0 b
SM of *B. bassiana* B16	28.66 b	7.8 b
SM of *M. anisopliae* M16	26.66 b	6.7 b
SM of *L. lecanii* L16	27.86 b	7.1 b

Numbers followed by different letters in the same column show a significant difference in the HSD test with an error level of 5%.

Table 4. Content of phenolic compounds qualitatively found in chili plants due to the treatment of secondary metabolites of four entomopathogenic fungi

Treatments	Tannins	Saponins	Hydroquinon
Control	+	+	+
SM of *B. bassiana* B10	++	++	++
SM of *B. bassiana* B16	++	+++	++
SM of *M. anisopliae* M16	+++	+++	++
SM of *L. lecanii* L16	++	+++	++

+ = a little; ++ = quite a lot; +++ = a lot.

Based on Table 3, each secondary metabolites of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 was able to affect root length or increase root length. Farahani-Kofoet *et al.* (2020) stated that the roots of plants infected by *F. oxysporum* would rot and cause the plants to collapse easily, so they are easily uprooted. Bani *et al*. (2018) and de Lamo & Takken (2020) stated that *F. oxysporum* could attack vessel tissue, thereby inducing root rot.

In addition, the application of each of the four entomopathogenic fungal secondary metabolites was able to extend plant roots. Lopez & Sword (2015) reported that *B. bassiana* promotes plant growth of cotton (*Gossypium hirsutum*). *B. bassiana* inoculation had a positive effect on plant growth parameters including root length of common beans (*Phaeseolus vulgaris*) (Afandhi *et al.,* 2019). Foliar inoculation of plants with the tested strains of *B. bassiana* and *M. anisopliae* increased plant height, leaf pair number, fresh shoot and root weights; however the increase was not always consistent across sampling dates (Jaber & Enkerli, 2017). The secondary metabolites producing enthomopathogenic fungi had been also reported as a plant tissue colonizer, plant growth enhancer, or as a naturally occurring endophyte (Ríos-Moreno *et al.*, 2016).

Effect of Secondary Metabolites of Four Entomopathogenic Fungi on Phenolic Compound Content. The qualitative tested tissue analysis was presented in Table 4. The results of the tannin compound test showed that the application of each secondary metabolite of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 produced more tannin compounds when compared to the control. It wes suspected that the treatment of four secondary metabolites of entomopathogenic fungi could increase tannin compounds in chili plants. The parameter of the amount of tannin content in plants could be seen in the presence of turquoise or blackish green.

This was in accordance with the opinion of Auwal *et al*. (2014), that a positive result on tannin testing was that the plant extract would be blackish blue due to the $FeCl_3$ reagent. The content of tannin compounds in the treatment of four secondary metabolites of entomopathogenic fungi was shown to have almost the same tannin content, and had good resistance to increasing the potency of tannin compounds compared to control.

Saponin test results showed that the application of each secondary metabolite *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 increased saponin content when compared to control (Table 4). This was in accordance with Juric *et al.* (2020), that the increase in phenol content due to the addition of antagonistic fungal metabolites. Plant content of secondary plant metabolites was affected by genetic, environmental, and agronomic factors (Neugart *et al*., 2018). Because the antagonistic fungal supernatant was absorbed by plants, substances that could be responsible for affected resistance give rise.

The results of the hydroquinone test on the treatment of four secondary metabolites of entomopathogenic fungi were more than the control (Table 4). Hydroquinone compounds were marked with a brick red color which could be seen in the chili leaf extract. Rahmania *et al*. (2018) confirmed that, the brick red color formed indicates the presence of hydroquinone. The presence of hydroquinone compounds in a plant would increase plant resistance to pathogen attack.

The increase in the content of these phenolic compounds (tannins, saponins, and hydroquinones) supported the observation of a longer incubation period and low disease intensity, even without disease symptoms (Table 2). Phenolic compounds were components of plant defense from within, which could be systemically induced through the application of entomopathogenic fungal secondary metabolites. Secondary metabolites that wre absorbed by plants would wake up the signal for phenol compounds to increase and function to overcome existing pathogens (Dangl & Jones, 2001).

CONCLUSION

The secondary metabolites of *B. bassiana* B10, *B. bassiana* B16, *M. anisopliae* M16, and *L. lecanii* L16 were able to inhibit growth of *F. oxysporum* f. sp. *capsici*, extend incubation periods, suppress disease intensity, increase plant height, and increase root length. It also increase the content of tannins, saponins, and hydroquinone compounds qualitatively compared to controls. The secondary metabolites of enthomopathogenic fungi could be used as organic control for soilborne pathogenic fungi.

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