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# APPLICATION OF RAW SECONDARY METABOLITES FROM FOUR ENTOMOPATHOGENIC FUNGI AGAINST CHILLI DISEASE CAUSED BY VIRUSES

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## ABSTRACT

***Application of raw secondary metabolites from four entomopathogenic fungi against chilli disease caused by viruses***. The purpose of this research was to investigate several kinds of raw secondary metabolits to decrease viral disease in chilli and inspect their side effect to plant growth. This research was conducted at experimental farm, Faculty of Agriculture, Jenderal Soedirman University from November 2018 to March 2019. The chilli seeds used for indicator plant were obtained from virus-symptomatic chilli. The raw secondary metabolites was collected from four microbial isolates used in this study, i.e. *Metarhizium anisopliae*, *Beauveria bassiana* (Papua isolate), *Lecanicillium lecanii* and *B. bassiana* Bio B10 (Jember isolate). The experiment was arranged in completely randomized design with five replications. Observation was performed on incubation period, disease intensity, AUDPC, germination percentage, plant height, number of leaves, and number of shoots. The result showed that raw secondary metabolites obtained from *M. anisopliae* gave the best capability to suppress disease development. Application of *M. anisopliae* raw secondary metabolites reduced incubation period, viral disease intensity as well as AUDPC in 34.22; 77.98 and 79.49%, respectively. The raw secondary metabolites of *L. lecanii* could increase percentage of germination, plant height, number of leaves, and number of shoots as 100; 38.96; 38.96 and 52.38%, respectively, compared to control.

**Key words:** chilli, entomopathogenic fungi, raw secondary metabolites, viral disease

## INTRODUCTION

Chilli is a horticultural crop that is quite important and widely cultivated, along the island of Java. Chilli pepper (*Capsicum annuum* L.) is a Solanaceae originating from South America (Perry *et al.*, 2007). National chilli pepper production tends to fluctuate each year. Chilli production recorded by the Central Statistics Agency, in 2017 was 1,206,266 tons, in 2016 a total of 1,045,601 tons, in 2015 a total of 1,045,200 tons, and in 2014 a total of 1,074,611 tons (Central Statistics Agency, 2019). Fluctuating chilli production every year can be caused by disruption in the chilli cultivation. One disruption in the process of chilli cultivation is caused by a pathogenic virus (Asare-Bediako *et al*., 2015; Devi *et al*., 2019). The viral disease on chilli can cause heavy production losses. According to A'yun *et al*. (2013), viral disease in chillies can reduce crop yields by 32–75% and according to Gunaeni & Wulandari (2010), crop losses range from 18.30–98.60%. Pathogenic viruses can be transmitted by seeds, for example tobacco mosaic virus (Sugiura *et al.,* 1975).

Conventional plant breeding techniques remain the major antiviral strategy thus far for the development of resistant chilli varieties (Thakur *et al*., 2018). Control of the viral disease in chili have been applied such as using medicinal plant extracts (Ahmed & Ram, 2016), botanical pesticides (Chaubey *et al*., 2017; Dougoud *et al*., 2019), and recently, the concern about viral DNA methylation, activation of gene silencing machinery and ubiquitination-mediated defense against begomoviruses have been reported (Thakur *et al*., 2018). Application of insecticides had been effectively used to manage this pest in the past, but it can develop resistance very rapidly (Bielza, 2008).

An efforts to overcome plant diseases in environmentally friendly way is the use of raw secondary metabolites (Soesanto, 2014; Rao *et al*., 2017). One of the raw secondary metabolites that can be used is obtained from entomopathogenic fungi (Molnar *et al*., 2010). Entomopathogenic fungi are one group of fungi that can be used as biological agents. The role of entomopathogenic fungi can also control plant diseases caused by viruses (Jaber & Salem, 2014). Indirect

biocontrol can affect endurance and increase plant growth. Plant protection genes produce pathogenesis-related (PR) proteins triggered by the colonization of squash rhizobacteria which act as a defense against ZYMV (*Zucchini yellow mosaic virus*). In addition, the increase in plant growth inoculated with *Beauveria bassiana* becomes more resistant to suppress ZYMV as has been done to protect tomato plants from *Cucumber mozaic virus* (Jaber & Salem, 2014).

The purpose of this study was to examine the best raw secondary metabolites of four entomopathogenic fungi for suppressing viral diseases in chilli and to examine the effect of its application on the growth of chilli plants.

## MATERIALS AND METHODS

**Research Site.** The research was conducted in the Plant Protection Laboratory and Experimental Farm, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto for five months, from November 2018 to March 2019.

**Preparation of Isolates**. Entomopathogenic fungi isolates used were *Metarhizium anisopliae*, *Beauveria bassiana* (Papua isolate), *Lecanicillium lecanii*, and *Beauveria bassiana* Bio B10 (Jember isolate). All isolates except Bio B10 were grown on PDA and incubated for at least 7 days at room temperature (Banu & Rajalakshmi, 2014).

**Multiplication of Isolates.** The entomopathogenic fungi isolates were propagated using broken corn media. The media was made by washing broken corns and submerged it in the water for 60 min, then drained thoroughly and air dried. The dried broken corn was put into plastic polypropylene bag as much as ± 100 g per plastic, covered with a stapler, then sterilized in an autoclave at 121 ºC and a pressure of 15 psi for 30 min. After cooling down, the sterlized broken corn was inoculated with the entomopathogenic fungi (*B. bassiana*, *L. lecanii* and *M. anisopliae*) separately, then incubated for 6–7 days (Mascarin *et al*., 2010).

**Preparation of Raw Secondary Metabolites.** Media mixture of rice washed water and coconut water was used for liquid substrate. The media was produced by mixing rice washed and coconut water in a ratio of 4 : 1 and ± 10 g $L^{-1}$ of sugar was added per liter of mixture. The mixture was boiled to dissolve the sugar, then the solution of 100 mL was put in an erlenmeyer flask of 250 mL. The flasks were cotton-puggled and autoclaved at 120 ºC for 20 min (Mascarin *et al*., 2010). The flask was inoculated with the entomopathogenic fungi thet multiplied on the broken corn separately. The broken corn media was washed with sterile water to release the fungi and the solution was inoculated into the flask (Alves & Pereira, 1989). The flasks then incubated in an orbital shaker (150 rpm) at 27 ± 0.5 ºC for seven days.

**Seed Treatment.** The chilli seeds was originally came from viral symptom chilli fruit in the field. The seeds were soaked in each raw secondary metabolites with conidia density of $10^6$ conidia $mL^{-1}$ for 30 minutes (Suryanto *et al*., 2010). Soaked seeds were drained and dried. Four replicates of 50 seeds then germinated on wet sterilized filter paper for 14 days saturated with 2 mL of sterile water (Samarah *et al*., 2016).

**Chili Seedlings.** Seeds that germinated on the filter paper were transferred to the seedling media composed by mixture of top soil and manure (1:1) (A'yun *et al*., 2013). The germination was remained for 14 days before planting.

**Planting Chilli Seedlings.** The seedlings were transplanted to the same media in a large polybags (± 3 kg) and managed with adding fertilizer and water until the maximum vegetative age. According to Kesumawati *et al*. (2018), at 15 days post transplanting (dpt) the symptom percentage of begomoviral infected plants is 3.1% and increasing to 65.1 and 89.9% at 30 and 45 dpt, respectively.

**Experimental Design.** This study used a randomized block design (RBD) repeated five times with the following treatments: control (sterile water), raw secondary metabolites of *Metarhizium anisopliae* (from density of $10^6$ conidia $mL^{-1}$), *Beauveria bassiana* (Papua isolate) (from density of $10^6$ conidia $mL^{-1}$), *Lecanicilium lecanii* (from density of $10^6$ conidia $mL^{-1}$), and Bio B10 (raw secondary metabolites of *B. bassiana* Jember isolate).

**Variables Observed.** The variables observed in this study were incubation period, disease intensity, germination level, number of leaves, and number of shoots. Incubation period and disease intensity were calculated use following formula (Gunaeni *et al*., 2014):

$$I = \frac{\sum (V.n)}{z.N} \times 100\%$$

I = disease intensity (%)
V = number of plants included in the scale of certain symptoms
n = scoring scores for specific symptoms
z = the highest severity score score
N = number of plants observed

The score of viral symptom attack is as follows:
0 = plants show no symptoms of virus (healthy)
1 = plants show mild mosaic symptoms
2 = plants show mosaic symptoms, the yellow groove is clearly visible (contrast)
3 = plants show mosaic symptoms, the yellow groove is clearly visible ( contrast) and there is a change in the shape of growth
4 = plants show all heavy yellow leaves, yellow grooves clearly visible (contrast), there is a change in the shape of growth, and dwarf plants

AUDPC (area under the disease progress curve) was calculated by (Louws *et al*., 1996):

$$AUDPC = \sum_{i}^{n-1} \left| \frac{Y_i + Y_{i+1}}{2} \right| \left( t_1 + 1 - t_i \right)$$

AUDPC = under the disease progress curve
Yi + 1 = observation data to i + 1
Yi = observation data to 1
ti + 1 = observation time to i + 1
ti = observation time to 1

**Data Analysis.** Data were analyzed by analysis of variance at 5% error level, then proceed with further DMRT at the same real level.

## RESULTS AND DISCUSSION

### Effect of Treatment on the Pathosystem Component

***Incubation period.*** The incubation period of the seeds showed no significant difference (Table 1). It is suspected that the condition of the used seeds was homogeneous and soaking time of the seeds into the secondary metabolites was not long enough, so the compounds in the raw secondary metabolites were less able to overcome the viral pathogens in the seeds. The soaking time the seeds into the raw secondary metabolites was only 30 min. According to Addrah *et al.* (2020), soaking the seeds in formulated flusilazole fungicide for 12 h was significantly reduced fungal contamination on the seeds. A method of soaking seeds in a 0–2% aqueous suspension of thiram for 24 h at 30 °C was able to eradicate infection by several fungi (Maude *et al*., 1969). In addition, the incubation period was not significantly different due to the ability of entomopathogenic fungi isolates and number of fungal conidia. This is in accordance with the opinion of Safavi (2010), that species of enthomopathogenic fungi has a large genetic variation among different isolates.

Nevertheless, the incubation period of the treatment which tended to be slower was the seed soaked in the raw secondary metabolites of *M. anisopliae* and *L. lecanii* as 34.22 and 18.71%, respectively, compared to control. This is accordance to the result of Farrag *et al*. (2015), that *M. anisopliae* alone or in combination with neem or L- Glutamic acid were no significant differences to control treatment, even the control was significantly higher than *M. anisopliae* treatment. The raw secondary metabolites produced in *M. anipsoliae* differ from other entomopathogenic fungi (Isaka *et al*., 2005).

Each treatment began to show viral symptoms at 21–42 days after planting. This is slower from Ali & Aprilia (2018) that the incubation period for diseases caused by viruses in plants between 10–15 days. Symptoms first appear on young leaves/shoots in the form of yellow spots around the bone of the leaf, followed by yellowing of the veins (vein clearing), concave and contracted with a light or yellow mosaic color. Severe symptoms make almost all young leaves or shoots bright yellow, and some are yellow mixed with green, concave leaves and contracted smaller and thicker.

Table 1. Statistic analysis on pathosystem component

| Treatment | Incubation period (dap) | Disease intensity (%) | AUDPC (%-days) |
|---|---|---|---|
| Control | 37.4 a | 3.18 a | 17.56 a |
| *M. anisopliae* | 50.2 a | 0.70 b | 3.60 b |
| *B. bassiana* | 39.2 a | 1.40 ab | 8.00 a |
| *L. lecanii* | 44.4 a | 1.47 ab | 7.42 a |
| Bio B10 | 34.2 a | 2.10 a | 14.65 a |

The number followed by the same letter in the same column shows the significantly difference with DMRT 5%.
dap = days after planting.

This symptom is in accordance to Subban & Sundaram (2012) and Devi *et al*. (2019), stated that majority of the plants showed severe leaf curling in both upward and downward directions with puckering, crinkling of leaves and in many cases with petiole elongation and complete sterility. Reduced leaf size, leaf distortion, stunting and blistering, dark green mottle and vein banding, defoliation and fewer and smaller fruits were the other characteristic symptoms. Chlorotic and necrotic spots and rings on leaves and apical necrosis.

***Disease intensity.*** The intensity of diseases caused by viruses in chili plants did not show any significant difference (Table 1). The diseases intensity of viruses in control plants was relatively small. This was suggested that the chilli seeds explored from the field with low virus infection and the virus did not transferred through the seeds. Thakur *et al*. (2018) stated that, some plant viruses are carried in the insect's feeding apparatus. *Chilli leaf curl virus* was transmitted by grafting infected chilli to healthy chilli (Mishra & Chauvey, 2018). However, *Pepper yellow leaf curl Indonesia virus* (PepYLCIV) is as a seed-transmissible virus in chili pepper plants (Fadhila *et al*., 2020).

The low disease intensity of each treatment including the control was suggested that the chili virus did not enter the seed embryo, so the virus did not carry over to the subsequent offspring. In addition, the low intensity of disease caused by a virus is thought to be caused by symptoms appearing when the plant is more than four weeks after planting. According to Gunaeni & Purwati (2013), differences in the intensity of attacks from each different treatment can be caused by increasing the number and size of leaves can affect the scoring of symptoms, thus directly affecting the size of the intensity of the attack. The greater the symptomatic plant infection and symptom score, the greater the intensity of the disease.

The intensity of the disease from each treatment showed different results (Table 1). The disease intensity from the treatment of *M. anisopliae*, *B. bassiana, L. lecanii* and Bio B10 tended to reduce the intensity by 77.98; 55.97; 53.77 and 33.96%, respectively, compared to control. The best decreasing disease intensity was the treatment of *M. anisopliae* which can reduce the disease intensity by 77.98% compared to control because of the compounds contained in raw secondary metabolites of *M. anisopliae*. *M. anisopliae* contains cyclopeptide, destruxin A (C29H47O7N5), destruxin B (C25H42O6N4), destruxin C, D, E, and desmethyl destruxin (Widariyanto *et al*., 2017).

***AUDPC (area under disease progress curve).*** All treatments gave lower AUDPC compared to controls (Figure 1). Raw secondary metabolites of *M. anisopliae* could reduce the diseases intensity compared to control. AUDPC was in line with the incubation period and disease intensity in the treatment of *M. anisopliae*. The treatment could be sorted from the most vulnerable that is control, Bio B10, *L. lecanii*, *B. bassiana* then *M. anisopliae*. From Table 1 showed that each treatment was able to reduce the AUDPC value of disease caused by viruses in the chili. The treatment of *M. anisopliae*, *B. bassiana*, *L. lecanii* and Bio B10 was able to reduce the value of the AUDPC by 79.49, 54.44, 57.74, and 16.57%, respectively, compared to control.

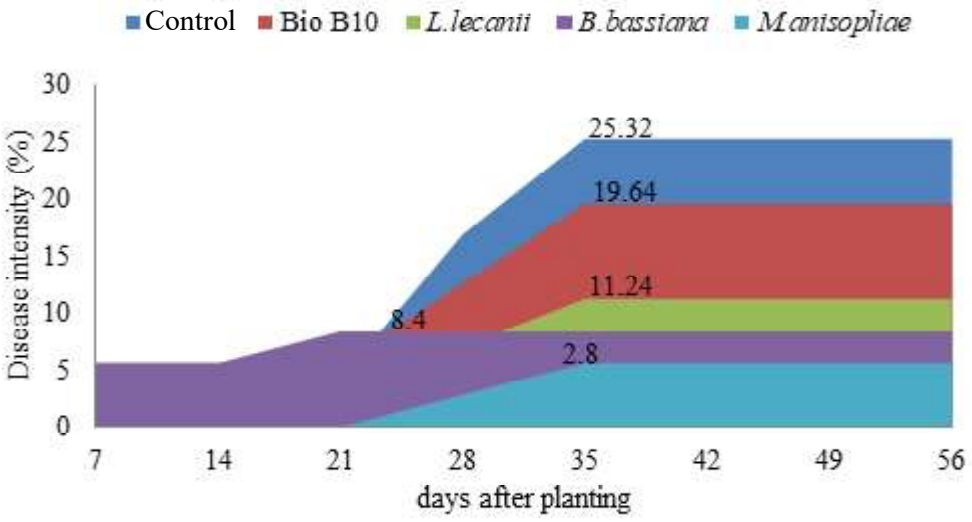


Figure 1. AUDPC caused by virus treated with raw secondary metabolites of four entomopathogenic fungi.

Raw secondary metabolites of *M. anisopliae* can reduce the AUDPC value because it contains several compounds, including starch degradation compounds, chitin decomposition, decomposition of fats and glycogen, antagonists to pathogenic fungi, chitinolysis, cytocalasin C and D (=zigosporin A), cyclodepsipeptide destrucin A, B, C, and D, L-prolil-L-leucine anhydride, L-prolil-L-valine anhydrid, and Desmetil destrucin B. *M. anisopliae* also secretes the enzymes lipase, chitinase, amylase, proteinase, pospatase and esterase (Gibson *et al*., 2014).

High AUDPC values in control are in line with disease intensity. This condition also applies to the seed covering with raw secondary metablites of *M. anisopliae* which has the lowest intensity value compared to all treatments (Table 1). According to Nuryani *et al*. (2011), the lower the AUDPC number, the more effective the treatment in controlling pathogens, and vice versa, the greater the AUDPC number, the more the treatment does not affect the pathogen infection. The raw secondary metabolites of *M. anisopliae* to chili seeds is thought to be able to reduce the disease intensity caused by viruses in the field, so that the AUDPC value obtained is lower.

**Effect of Treatment on Growth Components**

***Germination ability.*** The results showed that there were no significant differences in the germination ability of the chilli seeds, this could be suspected that the seeds maximally utilize the available water to carried out the imbibition process to support germination. Humphries *et al*. (2018) stated that, there are two factors that affect seed germination, namely the internal factors (seed maturity, seed size, dormancy) and external factors (water, temperature and light). Soaking seeds for 30 minutes can be stated as an effective soaking time for seed germination. In accordance to Suryanto *et al*. (2010).

***Crop height.*** The results of the statistical analysis showed that raw secondary metabolites of *L. lecanii* could affect the average plant height with the best results of 21.36 cm (Table 2) or increased crop height as 27.70% compared to control. The difference in plant height from each treatment was due to the ability to absorb nutrients from each plant and can also be caused by differences in the treatment given to each seed before planting. The treatments that were able to increase plant height were *L. lecanii* with percentages of 38.96% compared to control.

Raw secondary metabolites of entomopathognic fungi contain some compounds influenced plant growth because the fungi can be found in everywhere in the plant tissue. The fungi has also been reported as a plant tissue colonizer, plant growth enhancer, or as a naturally occurring endophyte (Lopez & Sword, 2015). Raw secondary metabolites of *L. lecanii* contain toxin namely bassionolidae, cyclosporine and dipicolinic acid which are insecticidal (Claydon & Grove, 1982; Khaerati & Indriati, 2015). These toxin compounds can prevent plants from insects, especially virus vectors, so plants can grow better (Khoiroh *et al.*, 2014).

***Number of leaves.*** The lowest number of leaves was observed from raw secondary metabolites of *M. anisopliae* as 18.93% or decrease as 18.05% compared to control and the highest number of leaves was from raw secondary metabolites of *L. lecanii* as 32.14% or 38.96% compared to control (Table 2). The difference in the number of leaves was caused by the availability and ability to absorb nutrients that are less optimal from each plant. In addition, it can also be caused by an infection from a virus that is in the chili seeds that inhibits the growth and growth of the number of leaves.

Raw seconday metabolites of *Lecanicillium* sp. exhibited direct plant growth promoting traits by production of indole-3-acetic acid and ammonia and by solubilizing inorganic phosphate and zinc. It also showed indirect plant growth promoting traits by producing siderophores and cell wall-degrading enzymes like, α-amylases, cellulases and proteases (Kumar *et al*., 2018).

Table 2. Analysis of growth component data

| Treatment | Germination ability (%) | Crop height (cm) | Number of leaves | Number of shoots |
|---|---|---|---|---|
| Control | 100a | 15.4 ab | 23.1 ab | 6.3 b |
| *M. anisopliae* | 96a | 13.5 a | 18.9 a | 5.9 b |
| *B. bassiana* | 100a | 17.2 ab | 26.1 ab | 7.3 ab |
| *L. lecanii* | 100a | 21.3 c | 32.1 c | 9.6 a |
| Bio B10 | 100a | 14.9 ab | 25.7ab | 7.2 ab |

The number followed by the same letter in the same column shows the significantly difference with DMRT 5%.

One part that is affected due to a viral infection is chloroplasts. Chloroplast is the main organelle that is attacked by plant viruses. The decreased rate of photosynthesis is caused by an abnormal form of chloroplasts, with a relatively smaller size and the amount of thylakoids in each grana which are decreased due to viral infection (Ali & Aprilia, 2018).

Disruption of plant growth such as plant height, number of leaves and leaf area due to virus infection can indirectly negatively affect physiological development in plants such as photosynthesis, especially in terms of utilizing sunlight as an energy source. Viral infections in general will reduce the total amount of chlorophyll as a result of reducing the efficiency of plant photosynthesis (Taufik *et al*., 2013).

***Number of shoots.*** Buds or shoots are part of plants that appear in the armpits of the leaves. Excessive shoots can interfere plant growth. The results showed a real difference among the seeds soaking treatment (Table 2). Raw secondary metabolites of *B. bassiana*, *L. lecanii*, and Bio B10 could increase the number of shoots by 15.87; 52.38 and 14.28%, respectively, compared to control. Raw secondary metabolites of *L. lecanii* gave the highest number of shoots and in line with the number of leaves (Table 2). This is in accordance to Kumar *et al*. (2018), that raw secondary metabolites contain production of indole-3-acetic acid and ammonia and by solubilizing inorganic phosphate and zinc inluencing the chilli growth component.

## CONCLUSION

The best raw secondary metabolites of four entomopathogenic fungi are from *M. anisopliae* indicated by incubation period, disease intensity, and AUDPC values as 34.22; 77.98 and 79.49%, respectively, compared to control. The best raw secondary metabolites influencing chilli growth are from *L. lecanii* indicated by germination ability, plant height, number of leaves, and number of shoots as 100; 38.96; 38.96, and 52.38%, respectively, compared to control.

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