El-Hayah Vol. 6, No.3 (2017) 104-111

JURNAL BIOLOGI

el – Hayah

Journal Homepage: http://ejournal.uin-malang.ac.id/index.php/bio/index
e-ISSN: 2460-7207, p-ISSN: 2086-0064

Potency of Endophyte Microbes as an Antagonistic Agent for *Colletotrichum capsici* Causes Anthracnose Disease in Cayenne pepper (*Capsicum frustescens*)

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DOI: 10.18860/elha.v6i3.5804

Article Info

Article history:
Received 01 December 2017
Received in revised form
15 September 2018
Accepted 02 October 2018

Keywords:
Trichoderma sp.
Bacillus cereus,
Colletotrichum capsici,
Capsicum frustescens

Abstract

Background: *An anthracnose disease caused by pathogenic fungal Colletotrichum capsici has been attacking the cayenne plants either harvested or has not been harvested. This disease must be handled appropriately and quickly because it can reduce the production of chili up to 90%. Recently, anthracnose disease prevention still use chemical fungicide that if applied for long time will cause new impact for environment.* ***Objective:*** *Trichoderma and Bacillus cereus endophytes may be used as antagonistic agents for C. capsici pathogens because they have various antibiotic compounds.* ***Methods:*** *This research uses experimental method. The stages of this study include sterilization of tools and materials, preparation of culture media of fungal and bacteria, rejuvenation of endophytic microbe culture Trichoderma sp. and Bacillus cereus, rejuvenation of C. capsici pathogen, antagonistic test in vitro using dual culture method.* ***Result and conclusion:*** *The results of in vitro antagonistic tests showed that inhibition percentage of Trichoderma treatment (96%) and combination treatment Trichoderma and B. cereus (97%) is not significantly different. While in B. cereus treatment (11, 88%) significantly different with all of treatments. Endophytes are shown by its dominating growth in petri dishes than C. capsici pathogen or B. cereus endophytes. Furthermore,for endophytes Trichoderma sp continued on in vivo test because it was most effective.The result for incubation period is 3 days after inoculation compared with negative control 2 days. For disease incidence 100%, and for disease intensity that is 61,25% compared with negative control equal to 88,75%.*

1. INTRODUCTION

Cayenne (*Capsicum frustescens*) is one of the main commodities of horticultural crops cultivated commercially in tropical areas of high economic value in Indonesia. Samsudin (2008) stated that the chilli requirement from year to year has been increasing in line with the increasing number of the growing population and the growing needs of the industrial sector using chilli as the basic material.

East Java has potential as a producer of cayenne pepper due to climate and environment that qualifies chili growing. Chili productivity figures in East Java did not increase significantly. Head of East Java Central Statistics Agency (2015) stated that cayenne pepper did not a significant increase in productivity compared to 2014 at 238.82 thousand tons, an increase of 11.33 thousand tons or 4.98 percent compared to 2013. Pests and diseases attack is one of the factors that inhibit in the cultivation of cayenne pepper (Setiadi, 2011).

One of the most important diseases in cayenne is an anthracnose disease caused by *Colletotrichum capsici* and *Colletotrichum gloeosporioides*. Plants affected by anthracnose disease are serious problems that can reduce chili production by 90% (Syukur, 2007). Piay *et al*., (2010) added that anthracnose disease can attacks chili both fruit before and after the harvest. The highest level of attack occurs during the rainy season.

Various efforts have been done by farmers to treat the problem of anthracnose disease such as using pesticides for the type of synthetic fungicide. Disease control using synthetic fungicides can cause negative impacts on the environment such as pathogen resistance, environmental pollution, and the death of non-target organisms. Until now, anthracnose disease control still uses synthetic fungicide as the main choice because it is considered to control the disease quickly and practically (Oka, 1995; Syamsudin, 2008).

he existence of increasingly advanced technology in agriculture, especially in the control of anthracnose disease in chili plants has made many antagonistic microorganisms developed as environmentally friendly biological control agents (Kusnadi *et al*., 2009). One of Biological controllers that use widely these days is Endophyic microbes. Endophytic microbes are microbes that live in plant tissue that is currently a lot of potential to be excavated. One potential of endophytic microbes is as a biological control agent for several diseases such as anthracnose disease. Endophytic microbes that can be used as anthracnose control are from *Trichoderma* and *Bacillus* groups.

Bacillus cereus bacteria is one of the endophytic microbes that has great potential to be used as a biological controller. This bacterium has a specific host, harmless to natural enemies of pests and other non-target organisms, and can be increased pathogenicity with genetic engineering techniques (Khetan, 2001). While *Trichoderma* sp. is a soil saprophytic fungus that is naturally a parasite that attacks many types of plant-causing fungi. *Trichoderma* sp. can become hyperparasitic in some types of plant-causing fungi, the growth is quick and does not become a disease for high-level plants (Trianto and Gunawan, 2003).

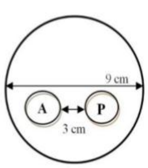
The effectiveness of *Bacillus cereus* and *Trichoderma* has been proven in several studies to control some plant diseases. The use of both antagonistic microorganisms aims to optimize the treatment of plant pathogens that are safe and environmentally friendly. Based on the above background, the researcher will conduct research on "Antimicrobial Trichoderma sp. and *Bacillus cereus* against pathogen *Colletotrichum capsici* causes anthracnose disease in cayenne pepper (*Capsicum frustescens*) ".

2. MATERIALS AND METHODS

This research was conducted in April-May 2017. In this study are three treatment with each treatment consists of six replications. The treatment consisted of endophytes *Trichoderma* + *C. capsici* on PDA media, endophytes *B. cereus* + *C. capsici* on PDA media, endophytes *Trichoderma* + endofit *B. cereus* + *C. capsici* on PDA media.

The materials used in this research are endophytes *Trichoderma* sp. isolated from strawberries, *Bacillus cereus* endophytes isolated from temulawak, the pathogenic fungi *Colletotrichum capsici* obtained from the Laboratory of Pests and Diseases of the Faculty of Agriculture University of Brawijaya Malang, Potato dextrose agar (PDA) media for fungi culture and nutrient agar (NA) media for bacterial culture, aquades, 70% alcohol, and chloramphenicol.

Antagonism treatment of *Trichoderma* and *B. cereus* endophytic in vitro using dual culture methods on PDA media. Inoculum with a diameter of 0.5 cm placed on a petri dish with a diameter of 9 cm. For each treatment made a diameter and given two points. The distance between them from the edge of the cup is 3 cm. The same way is also done in the combination treatment so that in the petri dish there are three points of culture. How to put the isolates as shown in Figure 1.



Explanation:
A: antagonism isolate
P: pathogen isolate

Figure 1. Laying of isolates for the antagonistic test in vitro (Szekeres *et al.*, 2006)

In Vivo Testing

Fruit chili sterilized with NaCl 0.5% for 5 minutes, washed with sterile water, then the surface sterilized with ethanol 70%. Next chili fruit soaked in a solution of antagonistic *Trichoderma* sp agent with 108 density konidia/ml for 2 hours, then given with 0.02% tween 80% (v/v) drops as reinforcement to the penetration. After 2 hours of continued with shed mould *C. capsici* as many as 20 μ l with the density of cell 1, 25x106 konidia/ml and 0.02% tween 80% (v/v) on the surface of the pepper that was already wounded by sterile needles ose. The entire treatment is placed in a sealed container at room temperature for 7 days. Furthermore, symptoms of antraknosa which consists of brown spots, the occurance of disease, and the intensity of the disease is being observed.

The parameters observed in this study are:

a) Incubation period (days): incubation period is the time it takes to do the pathogen infections, calculated based on the time of the first symptoms appeared on chilies after inoculation. Symptoms in the form of small patches of chilies and watery. The size of the wound can reach 3-4 cm on large chilies. Further attacks on the already severe, symptoms of the more obvious wound looks like a sunburned and wounds among flared until brown dark red to black (Craig, 2015).
b) The occurance of the disease (%) (Handayani, 2016):

The occurance of the disease = $\frac{n}{N} x 100\%$

With:
n: number of points of symptoms wound;
N: number of injuries observed point

c) The intensity of the disease (%) (Craig, 2015):

The intensity of the attack : $\left\{\frac{\Sigma(nxV)}{ZxN}\right\} x\ 100\%$

With:
n: number of fruit per class patches
V: rating score every class patches
N: number of fruit damati
Z: score the highest

Score spots disease classes used are as follows:

i. Score 0: no disease infection

ii. Score of disease 1: surface area of plants or plant parts are esophageal reaches 10%-25%
iii. Score of disease 2: surface area of plants or plant parts are esophageal is greater than 25%-50%
iv. Score disease 3: surface area of plants or plant parts are esophageal is greater than 50%-75%
v. Score diseases 4: dead plants.

3. Results and Discussion

Test of Microbe Endophytic Endorphic *Trichoderma* sp. and *Bacillus cereus* against *Colletotrichum capsici* by In Vitro

The results of antagonistic activity of microbe endophytic endophyte *Trichoderma* sp. and *Bacillus cereus* and a combination of both, indicate the potential for inhibition of the *Colletotrichum capsici*. The percentage value of endophytic endothelium inhibition of *Trichoderma* sp, *Bacillus cereus* and combination of both of *C. capsici* are listed in table 1.

Table 1. Percentage of endophytic endothelium inhibition of *Trichoderma* sp, *Bacillus cereus* and combination of both of *C. capsici*

Treatment	Percentage of inhibition (%)	Notation
Trichoderma sp.	96	ab
Bacillus cereus	11,88	a
Trichoderma + Bacillus cereus	97	ab

*the numbers followed by the same notation were not significantly different in the advanced test of duncan

The percentage of inhibition of pathogen *C. capsici* by optimum endophytic microbe is on *Trichoderma* sp treatment and *Trichoderma* + *B. cereus* combination with 96% and 97% respectively. For *Bacillus cereus* treatment the result is 11,88% lower than other treatment. Test of antagonism in vitro on *Trichoderma* sp it is seen that the mechanism of inhibition of mold against *C. capsici* pathogen is competition of space and nutrition. This is demonstrated by the growth of *Trichoderma* which dominates growth in petri dishes containing PDA media as in figure 2.

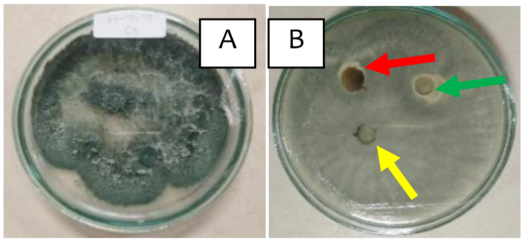


Figure 2. Treatment of *Trichoderma* sp. which antagonizes *C. capsici* grows dominating space in petri dishes containing PDA media; A: the appearance of the top of the cup and B: the appearance of the bottom of the cup. The red arrow: *C. capsici*, yellow arrow: *Trichoderma*, green arrow *B. cereus*

In Figure 2, it shows that the pathogenic fungi in the petri dish of its colony is covered by *Trichoderma* indicated by the green mycelia dominating the entire surface of the cup. Treatment with endophytic *Trichoderma* repeat one to six shows optimal inhibition of *C. capsici* pathogen because *Trichoderma* grows faster than its pathogen. According Volk and Wheeler (1983) that *Trichoderma* fungi have a fairly rapid growth rate, conidia are abundant, and can last long enough under adverse conditions. The mechanisms of antagonistic agents in inhibiting pathogens include competition, antibiotics, and parasitism (Amaria et al., 2015). *Trichoderma* sp which is obtained from strawberry endophytes inhibits pathogen by competition of space and nutrition as well as by way of parasitizing pathogens, this can be seen in microscopic observations of Figure 3, *Trichoderma* hyphae wrapped around the pathogenic *C. capsici* hyphae and causing pathophysiologic lysis.

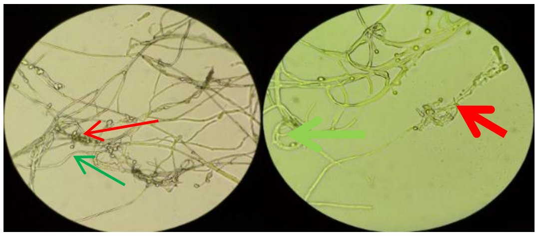


Figure 3. *Trichoderma* inhibition mechanism against *C. capsici* was observed under the microscope Nikon eclipse e200 400X magnification. **The red arrows** indicate abnormal hyphae (lysis) of pathogens and **green arrows** are *Trichoderma* hyphae that are wrapped around pathogens.

Trichoderma sp. is one of the effective biological control agents, can produce extracellular enzymes that make it possible for them to compete with other fungi (Rejeki, 2004). *Trichoderma*'s cosmopolitic nature is caused by this ability to produce a variety of secondary metabolites, resistant to inhibitors produced by other microorganisms, and this mold is relatively resistant to fungistatis (Eveleigh, 1985). Therefore, the percentage of inhibition on *Trichoderma* treatment in vitro is almost 100% in each repetition. The percentage of inhibition is said to be 100% if the antagonist agent covers the entire surface of the pathogen colony.

The second in vitro antagonism test is to antagonize the *Bacillus cereus* endophytic microbial with *Colletotrichum capsici* pathogen. Similar to the previous treatment, the observed percentage of inhibition was calculated on the seventh day after incubation. The percentage yield on *Bacillus cereus* treatment was different from the first treatment, because the percentage of inhibition was of little value. Thus *Bacillus cereus* bacteria have not been maximized in the fight against the pathogen *Colletotrichum capsici*, indicated by a small percentage of inhibition and also bacterial colonies and pathogens that both grow well in the cup as shown in figure 4.

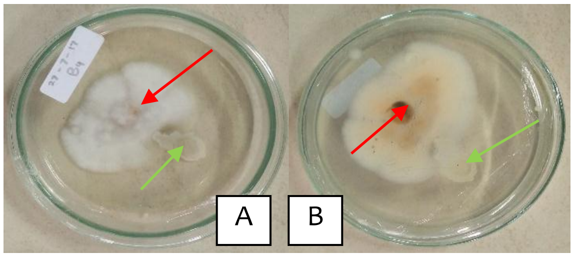


Figure 4. *B. cereus* antagonist treatment against *C. capsici*; A is the appearance of the surface of the cup and the appearance of the cup. The red arrow indicates *C. capsici* mycelia and green arrows show *B. cereus* colonies

Bacillus cereus may inhibit the growth of pathogenic *Colletotrichum capsici* on PDA media after incubation for 7 days although a smaller percentage compared with *Trichoderma* treatment indicates that the bacteria potentially as a biological agent. This is because the bacteria produce chitinase enzymes that can damage the cell wall of the pathogen. Muharni and Wijayanti (2011) stated that chitinase is an enzyme capable of decomposing chitin substances so that enzymes can degrade the chamber cell wall containing chitin. *Colletotrichum* hyphae wall has a microfibril texture made of chitin ie β-1,4-N-acetylglucosamine (Alfizar, 2013).

Test of Antagonistic *Trichoderma* sp. against *Colletotrichum capsici* in In Vivo

Based on the results of observation of the symptoms antraknosa pathogen on cayenne pepper fruit is colored basins form dark brown which is getting increasingly enlarged days on the surface of the fruit. According to the Nayaka et al., (2009) the big red chilies that stricken with antraknosa show symptoms in the form of an enlarged basin on the surface of fruit and in the central part of the basin there are black dots groups (Figure 5).

Figure 5 Results in vivo test in chilies that had been infected with the pathogen antraknosa, arrow indicates spot pathogen-infected pepper C. capsica.

The result of the antagonism in vivo test during incubation suggests that treatment using *Trichoderma*, disease symptoms occurred on the third day after inoculation compared with controls that the result shows that the emergence of symptoms of the disease on the second day after inoculation. The use of the endophyte *Trichoderma SP*. as antagonist has been able to inhibit pathogens in infected fruit cayenne pepper though in time not so long ago, that is three days after the the infection (table 2).

Table 2-test results of *Trichoderma* sp Antagonism against *Colletorichum capsici* in In Vivo

Treatment	Incubation period	Occurrencesof a disease	Intencitiesof a disease
Trichoderma sp. + patogens C. capsici in chilies	3 days	100%	61,25%
Patogens C. capsici in chilies (control)	2 days	100%	88,75%

The occurance of disease in treatment of *Trichoderma* and control is observed on the third day after inoculation alike showed symptoms of the disease antraknosa on 100% of pepper fruit visible with the appearance of brown spots on the point wounds made on the fruit. The severity of the disease observed by calculating the magnitude of the intensity of the disease in fruit that is given the treatment of *Trichoderma* result smaller 66.26% than on controls the intensity of the ailment of 88.75%.

The severity of the disease on the chilies on the third day after inoculation was 12.5%, on the fourth day after inoculation increased disease severity of 43.75% on the fifth day of the increasingly improved to 68.755% on the day the six increases be 81.25%, and on the seventh day after inoculation of the severity of the disease has reached 100% where all chili fruit rot pathogen *Colletotrichum capsici* due. Compared with controls, the value of the severity of the disease on the third day after inoculation of 68.75%, on the fourth day after inoculation increased to 75%, on the fifth day after inoculation increase to 100% thereby on the control negative rotten chili on the fifth day.

Pathogen inoculation was done by making the point wounds on chilies which had been soaked in the microbial suspension endophyte *Trichoderma SP*. result indicates that a pathogen infect the fruit on the third day after inoculation and show events disease at the point wounds the chilies are characterized by the presence of brown spots and the longer the more enlarged, with the decayed of the fruit as a whole. This is in accordance with the Nurmayulis et al., (2013) stating that the activity of boletus *Colletotrichum capsici* in pepper fruits can live through the wound. Mechanism of highly pathogenic *c. capsici involves* the production of enzymes degradation cell walls of plants, which are generally in the form of complex carbohydrates. In the first stage of pathogens secrete enzymes degradation polymers, named poligalakturonase, pectin lyases and also proteases that play a role in the process of initial infection and tissue maceration. In the second phase, the pathogens secrete enzymes α and β-galaktopirani-sidase and α-arabinofuranesidase which act degrades the polymer galaktan and araban for nutritional needs of pathogens. In addition to producing the enzyme *c. capsici* issued a toxin called kolelotrisin which can damage parts of the host cells and tissues, so that it can accelerate the damage to fruit and improves the process of respiration on the fruit.

The use of biocontrol agents such as *Trichoderma* allegedly can suppress the growth of pathogens in fruit, because it issued an extracellular enzyme biocontrol agent that kitinase enzymes capable of hydrolyzing β bonds between 1.4-subunit N-asetilglukosamina (NacGlc) on the polymer chitin as one component of the cell walls of Hypha of boletus in, so it can inhibit the growth of Hypha and suppress the rate of respiration (Wang et al., 2005) inhibition of respiration rate occurs at the time the alleged immersion or submersion of the fruit, the liquid into the cells of the epidermis of the skin of chili who opened the most so clogged and restricting the transport of O2 and CO2 are produced, as well as reduce or limit the evaporation water loss. Therefore at the treatment the severity of a rotten chili faster on control compared with soaked microbial antagonists.

The severity of the disease in the infected antraknosa chilies are observed on the third day after inokulais until the seventh day after inoculation. The intensity of the disease at the treatment by soaking agents antagonists showed lower results compared with controls, but after seven days of chili into a foul. This is in accordance with expressed by Semangun (1994) the chilies are stricken with antraknosa first shaped blackish-brown spots which later expanded into a foul rotten even soft dry. The symptoms appear, there are only partially rotten in the middle part of the base of the fruit, the fruit or the edge of the fruit.

4. Conclusion

Based on research conducted, the antagonism between *Trichoderma* sp. and *Bacillus cereus* and its combination of pathogen *Colletotrichum capsici* is the average percentage of inhibition for the treatment of *Trichoderma* sp. 96%; for the treatment of *Bacillus cereus* 11,88%; and for the combination of Trichoderma sp. + *Bacillus cereus* 97%. And the most effective endophytic microbes in inhibiting the pathogen *Colletotrichum capsici* are *Trichoderma* sp. The result in vivo test for incubation period is 3 days after inoculation compared with negative control 2 days. For disease incidence 100%, and for disease intensity that is 61,25% compared with negative control equal to 88,75%.

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