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Antagonist Microbes Potential Of Maize Root (*Zea mays* L.) To *Fusarium* sp. The Causing Of Fusarium Wilt Disease

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Abstract. This research is conducted to determine the potential and the endophyte fungi microbes antagonists mechanism and yeast in controlling of *Fusarium* sp. pathogen the causing of fusarium wilt disease. The research conducted in Sub-Laboratory of Disease, Department of Plant Pest and Disease, Faculty of Agriculture and Building Institute of Biosciences, Brawijaya University, Malang. The microbes isolation result of the maize root tissue is obtained by ten endophyte fungi genus and three yeast genus. The microbes fungi antagonist test result indicate that nine tested of endophyte fungi could suppress the growth of *Fusarium sp.* through 3 mechanisms of the antagonist namely mycoparasite mechanism, competition and antibiosis. The highest percentage of inhibition by *Trichoderma sp.* to *Fusarium sp.* The highest inhibition percentage by *Trichoderma sp.* (1 to *Fusarium sp.* is 52.22%. While the results of antagonistic yeast test indicate three yeasts tested not be able to suppress the growth of *Fusarium sp.* pathogen. The Scanning Electron Microscope (SEM) result succeed to indicate the mechanism of the micparasitic antagonist *Trichoderma* sp. fungi hyphae clearly stick and wrapped around fungi hyphae of *Fusarium sp.* fungi pathogen, thus causing the damage of hyphae structure.
Key words: *Endophyte fungi, Yeast, Fusarium sp. pathogen, Antagonist Mechanism, Scanning Electron Microscope.*

1. Introduction

Fusarium sp. pathogen fungi is a pathogen which cause fusarium wilt disease in maize crops besides downy mildew disease caused by the *Peronosclerospora maydis* fungi, leaf blight disease, leaf rust disease, and midrib rot. *Fusarium* is one of the most important pathogen fungi genus among other fungi groups which be able to affect the plants. According to [1], several species of *Fusarium* which found damaging to maize crops namely *F. oxysporum, F. verticillioides dan F. polidonogeum.*

Biological control effort has proposed as the substitute for plant disease control by using chemicals material. Unconsciously plants have associations with the microorganisms in the network with various mechanisms of resistance naturally, one of it is antagonist microbes. Microbes group are widely developed at present namely the microbes with endophyte and yeast fungi types. According to [2], endophyte fungi is an antagonist microbe which capable to produce antibiotic compounds which is active to fungi as well as pathogenic bacteria to human, animals and plants. Meanwhile, according to [3], yeast is the antagonist microbes in fungi group, saprophyte unicellular eukaryotic or parasite and has anti microbes more resistant to the environmental stress.

Hence, according to the description above it is necessary to research on non-pathogenic microbes of the maize crops roots which have potential as the controller agent of *Fusarium sp.* pathogen of the maize crops.

2. METHOD AND MATERIALS

2.1. Place and Time

This research is conducted in Sub Laboratory of Disease, Department of Plant Pest and Disease, Faculty of Agriculture, Brawijaya University and Building Institute of Biosciences Brawijaya University. The research started from February to July 2017.

2.2. Tools and Materials

The tools were used in the root sampling namely the small shovels, brown envelope paper, label paper, OHP, ice box. For the laboratory research, tools were used namely autoclave, laminar air flow cabinet (LAFC), petri dish (d =9 cm), media bottle, drop pipette, ose needle, tweezers, bunsen, spatula, scissors, erlenmeyer tube, handspayer, beaker glass, rotary shaker, glass and glass cover objects, camera microscopes, camera, Scanning Electron Microscope (SEM), and the identification books. The material were used namely maize root plant, Potato Dextrose Agar (PDA) medium, Yeast Extract Pepton Dextose (YEPD), 2% NaOCl solution, 70% alcohol solution and 96%, sterile aquades solution, antibiotic (chloramphenicol), tissue sterile, plastic wrapping, aluminum foil, paper label, spirtus, matches.

2.3. Endophyte Fungi Isolation and identification

The method using for the isolation of endophyte fungi on the root plant part is based on the method which described by [4], namely the root sample washing in the water flow, then take the root which has been cut ± 5 cm. The surface sterilization doing by soaking the root pieces in 2% NaOCl solution for 1 minute, then rinsed with 70% alcohol solution for 1 minute and rinsed with the sterile aquades for 1 minute as twice, then dried up on the sterile tissue. Sterilized root samples were cut into ± 1 cm size by using the sterile scapel and then planted on the 9 cm petri dish containing PDA medium with 3 root samples method in each petri dish. The isolates are then incubated at the 25-30°C for 5-7 days or until the fungi growing to fill the petri dish (full plate).

The observation is conducted macroscopic and microscopic which the result then used for identification based on the *Illustrated Genere of Imperfect Fungi fourth ed* [5] identification guide book. The macroscopic observation conducted by observing the macroscopic morphological appearance of the fungi colonies, pattern dispersion of colonies in petri dish (concentric and non-concentric), colony texture and the duration required by the colony to fill the petri dish (*full plate duration*).

Microscopic observation conducted by observing the morphological appearance of fungi colony by using the microscope which encompass the presence or absence of septa in hyphae, hyphae growth, hyphae color, presence or absence of conidia, conidia color, conidia form, and conidia dispersal pattern. Observations conducted by using 400 x (40 x 10) magnification.

2.4. Endophyte Fungi Isolate Antagonist Test and *Fusarium* sp.

In order to indicate the fungi potential as the antagonist then conducted the petri dish observation by using the artificial PDA medium in accordance to [6], the inoculated fungi isolate in one of the petri dish edge with the 9 diameter and 3 cm distance position faced with the isolate pathogen to be tested (Figure 1). Thus would be obtain a combination of testing between the *Fusarium sp*. pathogen and the fungi isolates. The treatment is consistent with the number of fungi isolate which obtained and control (without antagonist fungi). The experiment purpose in this step is to perceive the presence of inhibition as well as the magnitude of fthe fungi isolates resistance to the *Fusarium sp.* growth.

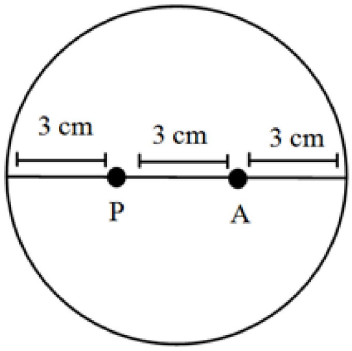

Figure 1. The Inoculated Fungi Isolate

Description :
P = *Fusarium* sp. Pathogen
A = Endophyte Fungi

Then it incubated at the room temperature, the colony pathogen growth radius is measured every day for 7 days of observation. Inhibition of the colony growth isexpressed in percentage which is calculated by the (Van den Heuvel, 1970)[7] formula, namely:

$$I = \frac{r1 - r2}{r1} \times 100\%$$

Explanation :
I : Inhibition Percentage
r1 : Radius of the pathogen colony which the growth direction is opposite with the fungi (cm).
r2 : Radius of the pathogen colony which the growth direction is approached with the antagonist fungi colony (cm).

2.5. Yeast Isolate Antagonist Test with *Fusarium sp.* Pathogen

Yeast antagonist test method in-vitro in accordance to Shofiana et al. (2015) (Figure 2) [8]. The yeast is scraped on the PDA medium right in the middle of the petri dish perpendicularly for 1 inoculation loop. *Fusarium sp.* pure cultures is taken by the stopple drill and place it on the right and left side of the yeast scratch with the ± 3 cm distance and then incubated at the room temperature. Width of inhibition zone observations and the inhibition level percentage is relatively yeast to the *Fusarium sp.* fungi which conducted every day. The control treatment without yeast inoculation also prepared as the comparison.

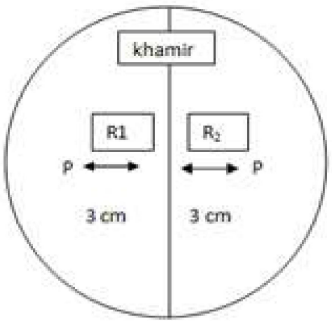

Figure 2. Yeast antagonist test method in-vitro

Inhibition level percentage to the pathogen is calculated by the following Hadiwiyono (1999) [9] formula, as follows:

$$\text{THR} = \frac{dk - dp}{dk} \times 100\%$$

Explanation :
THR : Relative inhibition level percentage to the pathogen growth
dk: The number of colony radius (r1 + r2) of pathogen without treatment of yeast (control)
dp: the number of colony radius (r1 + r2) of the treated yeast pathogen

2.6. Antagonist Mechanisms Observation with Scanning Electron Microscope (SEM)

The method in accordance to Hastuti (2016) [10] namely, the antagonism mechanism is observed by making preparat by slicing 2x2 mm of interacting fungi colony and placed on the sterile glass cover. Then conducted a multilevel drying or dehydration by using the 30%, 50%, 70%, 80%, 90%, dan 96% ethanol solution by spraying directly to the fungi isolates. Spraying conducted with ± 5 minutes distance.

After the isolates dried then it is placed on the specimen holder device (aluminum stub) with the colloidal silver paste adhesive and coated with gold metal (Au) (metal thickness ± 15 mm) by following the further evaporation process observed by using electron scanning microscope. The scanning electron microscope way working is the rays of light emitted on the lens condenser, before entering the condenser lens there is an emitted beam of electrons regulator. The light passing through the objective lens extended to the specimen which is tilted on its capture, this specimen is illuminated by the x-ray detection which produces an image forwarded on the monitor screen. Observations made visually to the photomikograf results are processed with the Fuji film black and white photograph.

3. Data Analysis

The microbes isolates of endophyte fungi and yeasts is identified of the maize plants rooting analyzed descriptively and displayed in the form of images based on the macroscopic and microscopic appearance. Experiment design conducted in vitro antagonist test namely Complete Random Design (CRD).

The obtained data from the microbes antagonistic test of endophytic and yeast fungi with *Fusarium sp.* were analyzed by using the variance (Anova) analysis and continued with the Duncan test with 5% level if there is a real difference.

4. Results and Discussion

4.1. Maize Crops Symptom stricken by the *Fusarium* sp. pathogen in field

Symptom which appear in the base of stem on maize crops through a changes color to brown by the putrefaction. There is the mass as white spores around leaf and the base of stem.
According to Sastrahidayat (2010) [11], the beginning symptom of this disease attack is the occurrence of leaf bleaching and the leaf midrib, as following of the ducked leafstalks. The withered leaves would gradually turn yellow. Leaf withered occurs from the bottom and continue to the upper leaves.

4.2. Isolation and Identification Result of *Fusarium sp.* Pathogen Fungi

The macroscopic characteristic of *Fusarium sp*. namely the colony has white color, there is the white bones in the center of colony. The lower colony has yellowish white. Types of concentric is dispersion, round with the rather coarse colony surface texture as cotton, the density is rather tight, and the thickness is rather thin. The diameter at 7 days age is 6 cm.

Fusarium sp. microscopic is obtained from the sectional and slim hygiene hiala result. Hyaline conidiofor is sectional and unbranched. Hyalkonidia hyaline, not insulated with a thin wall, and oval-shaped with slightly pointed tip. Microconidia shaped is as the crescent moon or the hook with a pointed tip, hyaline, and has 3 to 5 barriers. The macroconidia size obtained is 19.34µm x 2.55µm. Macroconidia form of *Fusarium sp.* is as the crescent moon, hyaline, and sectional.

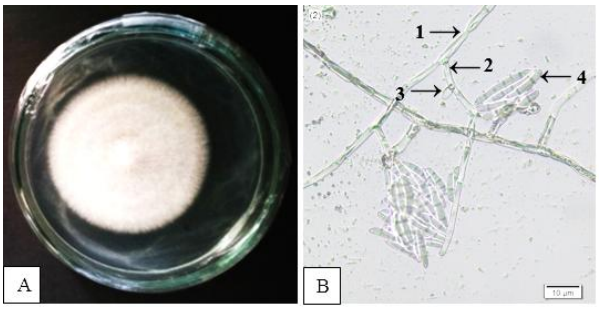

Figure 3. *Fusarium* sp. microscopic fungi (400 times magnification)

Sastrahidayat (2010) [11] mentioned that *Fusarium* sp. has a macroconidium in the form of curved, long end with a small tip and has one or three bulkheads, while the microconidium has a non-sectional or one-sided shape and is produced by the sporodochium (its size is smaller than macro).

4.3. The Endophyte Fungi Microbes and Maize Root Yeast Isolation and Identification Result

After the isolation, purification and identification step then found 10 endophytes fungi and 3 yeasts namely: *Nigrospora* sp. endophyte fungi, *Alternaria sp*., Root Fungi (isolate 1), *Phoma* sp., *Curvularia sp. (*isolate 1), *Fusarium sp*. 1, *Culvularia sp*. 2, *Fusarium sp*. 2, *Trichoderma sp*.1, and *Trichoderma sp.* 2. Thus, *Trichoderma sp*. 2, and *Candida sp*. yeast, *Metschnikowia* sp., and *Pichia sp.*

4.4. Endophyte Fungi Antagonist Test Result to *Fusarium* sp. Pathogen

The antagonist test result of the Endophyte Fungi to *Fusarium* sp. pathogen which conducted in vitro by using the direct opposition method on the PDA medium that presented in table 1.

Table 1. The mean percentage of the fungi isolates inhibition to Fusarium sp. for 7 HSI (days after the isolation).

Endophyte Fungi Isolate Treatment	Growth power inhibition (%)	
Control	0	a
Phoma sp.	16,8	b
Curvularia sp. (isolate 1)	24,33	bc

Root Fungi (isolate 1)	26,79	bc
Nigrospora sp.	28,77	c
Curvularia sp. (isolate 2)	32,25	c
Alternaria sp.	32,41	c
Trichoderma sp. (isolate 2)	50,83	d
Trichoderma sp. (isolate 1)	52,22	d

The mycoparasite antagonist mechanism and competition is indicate by the *Trichoderma* sp.1 and *Trichoderma* sp.2 fungi isolates. The highest inhibition percentage by *Trichoderma* sp.1 to *Fusarium* sp. pathogen is 52,22%.

4.5. *Trichoderma* sp.(isolate 1) Fungi

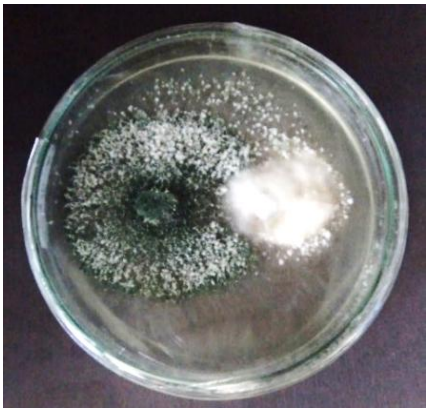

Figure 4. Antagonistic test of *Trichoderma* sp. (isolate 1)

Inhibition level differences to Fusarium sp. pathogen by antagonist fungi is allegedly closely related to the ability of the antagonist fungi to compete with the pathogens primarily as a microparasite and the growing speed. The antagonist fungi test result indicate that eight of the tested fungi microbes could suppress the growth of *Fusarium sp.* through three mechanisms of the antagonist namely mechanism mycoparasite, competition and antibiosis.

According to Sastrahidayat *et al*., 2015 [12], *Trichoderma sp.* fungi have the ability to grow rapidly to fulfil the petri dish for 3 days to be able to compete with the fungi pathogen in obtaining the nutrients by means of antagonistic fungi surround, blocking and then grow in the pathogen fungi hyphae.

For furthermore, there are four isolates in the antagonist test with competition mechanism namely *Alternaria* sp. fungi, root fungi 1, *Culvularia* sp. fungi 1, and *phoma* sp. fungi. According to Zuhria *et al*., 2016 [13] antagonist mechanism of the endophyte fungi has the ability in obtaining the space and nutrients as well as the enzyme production to counter pathogens cell component. Mechanism of the endophyte fungi competition does not as targeting the pathogens directly, however through the physiological and metabolic changes secondary. It is also supported by the statement of Mukarlina *et al.* (2010) [14] in the PDA medium, the presence of antagonist fungi causes the limited site of growth and nutrients for pathogen fungi growth. Competition which occurs in the double culture method is due to the need for nutrients such as carbohydrates, proteins, essential amino acids, and other nutrients.

Antibiosis mechanism is produced by *Nigrospora sp*. and *Culvularia sp.* fungi treatment. This is indicated by the clear zone between the tested endophyte and pathogen fungi. Endophyte fungi is be able to release the antibiosis substances which be proved by the no growth of pathogens in the clear zone medium. According to Hallmann et al., 2001[15], the endophyte fungi has an antagonistic mechanism characterized by the clear zones around endophyte and pathogenic fungi. As well as Arnold et al., 2003 [16] describes that the endophyte fungi has direct mechanism in suppressing pathogens, namely through the production of antibiotic and lytic enzyme secretion.

4.6. The Yeast Antagonist Test Result To *Fusarium* sp. Pathogen

The antagonist test result between yeast to *Fusarium* sp. pathogen conducted in-vitro on the PDA medium which presented in table 2. as follows:

Table 2. The mean percentage of the yeast inhibition to Fusarium sp. pathogen for 7 HSI (days after the isolation).

Yeast Isolate Treatment	Growth power inhibition (%)	
Control	0	a
Candida sp.	2,31	a
Metschnikowia sp.	6,52	a
Pichia sp.	8,31	a

There is no interaction of the yeast colony to *Fusarium sp.* fungi pathogen hyphae on the PDA medium surface from the observation result, but there is only space and nutrition competition by the *Fusarium sp*. pathogens growth. This is indicated in the *Candida sp.* and *Metschnikowia* sp. yeast treatment namely the *Fusarium* pathogen growth more control the space and nutrients in petri dish.

There are factors which affect the *Fusarium sp*. antagonist test such as pH medium and antagonist test environmental pressure. It affects the microbes activity in application as the biocontrol agent. According to Mohamed and Haggag (2007) [17] mentioned that in bicontrol strain application planning, the factors such as pH, low temperature, moisture be able to affect the growth and metabolite of the biocontrol agents.

Besides the competition mechanism other things indicate in the *Pichia* sp. yeast treatment. The antibiosis mechanism indicate the appearance of inhibition zone and the color changes of the *Fusarium sp.* pathogen fungi base colony. Formation of the secondary metabolite compounds be able to cause fungistatic, cell wall lysis, or necrotic, so the growth of pathogenic fungi be inhibited. Its accordance to Masih *et al.* (2001) [18] statement that *Pichia membranifaciens with B. Cinerea* pathogen yeast antagonist test causing the appearance of inhibit zone around the yeast meanwhile the hyphae of pathogen failed to grow in it zone. Then the pathogen colony color changes caused by compounds or enzymes that be able to lysis the cell wall.

4.7. *Trichoderma* sp Fungi Antagonist Mechanism Observation Results to Fusarium sp.

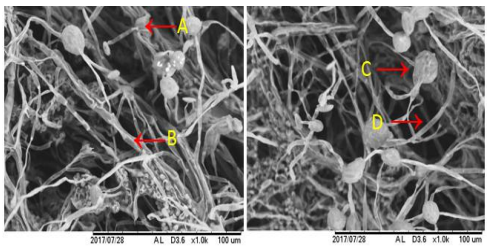

Figure 5. *Trichoderma* sp Fungi Antagonist Mechanism Observation Results to Fusarium sp

SEM (Scanning Electron Microscope) is clarify the mechanism of antagonism between *Trichoderma sp.* and *Fusarium sp.* indicate that *Trichoderma* sp. hyphae sticking and wrapped around *Fusarium* sp. hyphae thus causing the hypha structure damage. *Fusarium* sp. hyphae through a structure damage due to its flat structure by the *Trichoderma* sp. hyphae mycoparasite and nutrients in the *Fusarium* sp. absorbed by the *Trichoderma* sp. fungi hyphae. According to Benitez *et al.* 2004 [19] mentioned that *Trichoderma* be able to sticking on the pathogen by binding, rolling the pathogen cell walls and forming the appressorium. The mycoparasite process involved the pathogen morphology as twisted and the appresorium structure forming functioning to through the pathogen wall. In addition, according to Sastrahidayat *et al.* 2015 [12], mycoparasite process occurs by the direct interaction of each antagonist fungi hyphae with pathogen fungi hyphae. *Trichoderm* hyphae seen as wrapped around hyphae of the pathogen fungi.

5. Conclussion

- ✓ There are fungi and yeast microbes which were succeed in isolation of the maize rooting namely 10 genera of endophyte fungi that have been identified such as, *Nigrospora sp., Alternaria sp., Fungi Root 1. Sclerotium sp., Curvularia sp.1, Curvularia sp.2 Fusarium sp.1, Fusarium sp.2, Trichoderma sp.1* and *Trichoderma sp.2* and the three yeast genera which identified as *Candida sp., Metschnikowia sp.,* and *Pichia sp.*
- ✓ Eight isolates of antagonist tested fungi microbes by antagonists were be able to inhibit the growth of *Fusarium sp.* with antibiosis mechanism, competition and mycoparasite. The most potential fungi microbes to suppress the growth of *Fusarium sp.* is the Trichoderma sp. 1 and Trichoderma sp.2. fungi, while the three isolates of yeasts have not been able to inhibit the growth of Fusarium sp.
- ✓ The observation results by the SEM (Scanning Electron Microscope) indicate the mechanism of the *Trichoderma sp.* fungi antagonism with *Fusarium sp.* fungi pathogen is the mycoparasite mechanism

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