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Antagonist Assay and Molecular Identification of Soil Molds Antagonist to Pathogenic *Fusarium* on Tomato Plants (*Lycopersicum esculentum* Mill.) in Bocek East Java Tomato Field

Herlinda Mawardika[1], Suharjono[1]

[1]Department of Biology, Faculty of Mathematics and Natural Science, University of Brawijaya, Malang, Indonesia.

Abstract: One of the problems in tomato cultivation was wilt disease caused by pathogenic *Fusarium*. The effective solution was using antagonist molds as biological control agent against pathogenic *Fusarium*. The purposes of this research were to determine potency of antagonist molds and to identify potential antagonist mold to inhibit growth of pathogenic *Fusarium*. Pathogenic *Fusarium* were isolated from tomato stems, while antagonist molds were isolated from soil sample. Screening of isolates and antagonist assay was carried out using dual culture method, then continued with non-volatile metabolite assay. Percentage of inhibition zone was analyzed by One-way ANOVA at a significant level of $\alpha = 0.05$ by SPSS. Mold isolates were identified based on *Internal Transcribed Spacer* (ITS). Isolate FB.1 and FB.2 were obtained from tomato stems. Three soil molds, KT.7, KT.10, and KT.16 have antagonistic activity to pathogenic *Fusarium*. KT.16 colony has the highest antagonistic activity against isolate FB.1 (59.84 %), whereas isolate KT.10 against isolate FB.2 (54.67 %). Metabolites of isolate KT.16 showed the highest inhibition percentage against FB.1 and FB.2 isolates (34.82 and 33.67 %). Isolate KT.16 was the best antagonist mold and was identified as *Trichoderma longibrachiatum* ATCC 52326 with similarity 100 %.
Keywords: antagonist mold, *Fusarium*, inhibition, tomato, *Trichoderma.*

Introduction

Tomato plant is one of the cultivated plants in Indonesia. Tomato has sour taste and contains vitamin C to enhance human immunity. Tomato is used in food industries as food coloring and flavor enhancer. Based on Central Bureau of Statistics, national tomato production in 2014 reached 895.163 ton[1]. The major problem in tomato cultivation is wilt disease caused by *Fusarium,* especially *Fusarium oxysporum* f. sp. l*ycopersici*. *Fusarium* wilt disease reduces tomato productivity up to 80 %[2]. Tomato plant is infected by *Fusarium* through root system. This mold colonize vascular system and inhibits water transport toward upper side of plant[3].

Fusarium produces three type of asexual spores. There are microconidia, macroconidia, and chlamydospores. *Fusarium* has microconidia which produced on monofialides. Generally chlamydospores are single, double, or in chains[4]. The main characteristics of *Fusarium oxysporum* are colony colour, pigmentation, and the presence of asexual spores. Colony colour and pigmentation are white and peach to dark violet. Macroconidia are straight and relatively slander or slightly curved and thick with 3-7 septate[5]. *Fusarium* wilt

disease causes the older leaves on one side of the plant turns yellow and buckle at their petiole. Large portion of the xilem turning brown and become die soon[6].

Controlling pathogenic mold using chemical pesticide is not effective. Pesticide alters the soil quality since it decrease soil ability to support crop production and decrease of beneficial soil microflora[7]. Microbes isolated from rhizosphere are able to adapt easily and control pathogen in root and leaves of plants[8]. Some potential antagonist molds known as biological control of pathogenic *F. oxysporum* are *Gliocladium, Penicillium, Trichoderma*, and *Aspergillus*[9]. Mechanisms in controlling pathogen growth including nutrition and space competition, microparasitism, induced systemic resistance, and antibiosis[10].

Fusarium wilt disease still become serious problem in tomato cultivation and its important to find potential antagonist mold. The objectives of this research were to determine potency of antagonist molds and to identify potential antagonist mold to inhibit growth of pathogenic *Fusarium*

Experimental

Isolation of *Fusarium* and antagonist molds

Fusarium isolates were isolated from infected plant stems. Infected plant stem was sliced into pieces (± 3 mm). Stem slices were surface sterilized with 5.25 % NaOCl for 30 second and subsequently rinsed with three steril distillated water for 1 min. They were placed on Potato Dextrose Agar (PDA) medium containing 50 ppm streptomycin and incubated at 30 °C for five days[11]. Antagonist molds were isolated from field soil in Bocek Village, Karangploso subdistrict, East Java, Indonesia. Soil sample was serial diluted in NaCl 0.85 % up to 10^{-7}. Each suspension (0.1 ml) was poured plate using PDA medium containing 50 ppm streptomycin, then incubated at 30 °C for 5 days. Mold isolates were purified by single spore inoculation[12]. The total number of molds were determined based on *Total Plate Count* method.

Screening of antagonist molds

Mycelial disc (3 mm) of soil mold and *Fusarium* culture was placed on PDA at a distance 3 cm. *Fusarium* disc was placed on another petri dish as control. Plates were incubated at 30 °C for 3 days[13]. Percentage of *Fusarium* inhibition was recorded and calculated based on following equation.

$$L = \frac{(C-T)}{C} \times 100$$

L : percentage of inhibition
C : diameter of *Fusarium* as control (mm)
T : diameter of test *Fusarium* at dual culture (mm)

Antagonist assay using dual culture method

Single spore of *Fusarium* and antagonist molds isolates were placed on PDA medium at a distance 3 cm and incubated at 30 °C for 6 days. Single spore of *Fusarium* was inoculated on another petri dish as control. The experiment was carried out with three replications[12]. Percentage of *Fusarium* inhibition was recorded for 6 days and analyzed using One-way ANOVA (Analysis of Variance) at a significant level of $\alpha = 0.05$ by SPSS.

Non-volatile metabolite assay

Non-volatile metabolites assay of antagonist molds has been described for determination antifungal activity[14]. Three mycelial discs of five days antagonist molds culture (3 mm) were inoculated into 100 ml Potato Dextrose Broth (PDB) and incubated at 30 °C on rotary shaker at 120 rpm for 12 days. Mold culture was filtered using Whatman Filter Paper No.1 and sterilized by membrane filter (0.2 µm). Culture filtrate was poured into melted PDA medium to obtain final concentration 10 % (v/v). The medium was poured into steril petri dishes. Mycelial disc of five days *Fusarium* culture was inoculated at the centre of PDA medium. The experiment was carried out with three replications. Percentage of *Fusarium* inhibition was recorded and calculated based on equation 1, then data were analyzed using One-way ANOVA (Analysis of Variance) at a significant level of $\alpha = 0.05$ by SPSS.

Molecular identification of *Fusarium* and antagonist mold

Three mycelial discs of antagonist molds and *Fusarium* were inoculated in Potato Dextrose Broth and incubated on rotary shaker at room temperature for 3 days. Isolation of DNA was carried out based on modified method[15]. DNA sequences were amplified by *Polymerase Chain Reaction* (PCR) using primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)[16] based on PCR reaction condition as in Table 1. PCR product was visualized by electrophoresis using agarose gel 1.5 % and O'GeneRuler^{TM} DNA Ladder Mix 100 as DNA marker, then stained with ethidium bromide[17]. DNA was purified and sequenced using the same sets of primers in First BASE, Malaysia. DNA sequences were compared with those available in NCBI using BLAST and aligned by software. Phylogeny tree was constructed using MEGA 5. Evolutionary distances was determined based on Maximum Likelihood algorithm with Tamura-Nei method[18].

Table 1. PCR reaction condition for DNA amplification

No.	Reaction	Temperature (°C)	Time (min)
1	Pre-Denaturation	95	10
2	35 cycles: Denaturation	94	0.25
3	*Annealing*	55	0.5
4	Extension	72	0.25
5	Extension	72	7

Result and Discussion

Antagonist molds of pathogenic *Fusarium*

There were 19 candidate of antagonist molds from soil sample and two *Fusarium* isolates (FB.1 and FB.2) from infected plant stems. Antagonist molds were determined based on inhibition percentage over 30 %. Inhibition activity was characterized by reduction in colony diameter of pathogen. Based on screening results, isolate of soil molds KT.7, KT.10, and KT.16 showed antagonistic activity against isolates FB.1 and FB.2. This result was used to determine the presence of antagonist potency of molds in order to inhibit growth of pathogen[19].

Potency of antagonist molds against pathogenic *Fusarium*

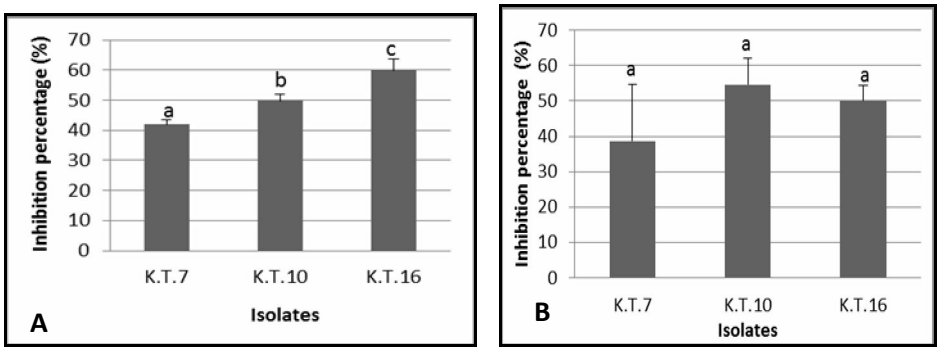


Figure 1. Inhibition percentage of *Fusarium* by antagonist molds

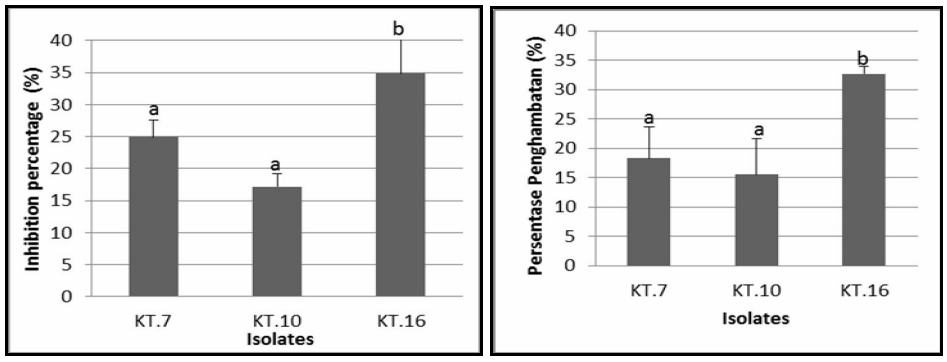
Isolate KT.16 had the highest inhibition (59.84 %) against *Fusarium* isolate FB.1, Isolate of soil molds KT.7 and KT. 10 showed inihibition percentage 41.99 and 49.77 % which significanly different (Fig. 1A). *Fusarium* influenced antagonist activity of molds. Isolate KT.7 resulted maximum inhibition against *Fusarium* isolate FB.2. *Fusarium* inhibition percentage of isolates KT.7, KT.10, and KT.16 were 38.67 %, 54.67 %, and 50 % respectively (Fig. 1B). Antagonistic activity of *Trichoderma* have been reported. Percentage inhibition of *T. longibrachiatum* against *Fusarium oxysporum* was 50.04 %[20]. Some isolates *T. asperellum* inhibited mycelia growth of *F. oxysporum* f. sp *lycopersici* around 23-71 % after 6 days incubation[21], so this result was moderate.

It has been reported that molds which control pathogen were *T. harzianum*, *T. asperellum*, and *A. flavus*[22]. The fast growth of antagonist molds indicated their ability to compete for nutrition and space. Early research also stated that *Nigrospora* sp, *Penicillium* sp, *T. harzianum*, *T. viride*, and *G. virens* were able to reduce *Fusarium* wilt disease caused by *F.* o*xysporum* f. sp *lycopersici* and *F.* o*xysporum* f. sp *cucumerinum*[23].

Trichoderma was known as effective biocontrol agent since involving some inhibition mechanism, such as competition for space and nutrition, induced plant resistence against pathogen, and antibiosis[24]. Another mechanism was mycoparasitism. Antagonist mold produces lytic enzyme. This enzyme will degraded cell wall of pathogen which results oligomer inducing mycroparasitism. Antagonist mold grows toward pathogen, adhere, and form appresoria[25]. Then cell wall degrading enzyme such as chitinase, protease, and glucanase will be released, allowing this mold enter the lumen of pathogen and use hypha component as nutrition source[26].

Potency of non-volatile metabolite of antagonist molds against pathogenic *Fusarium*

Culture filtrate of isolate KT.16 had the highest inhibition against both *Fusarium*. Inhibition percentage of antagonist molds against *Fusarium* FB.1 approximately 17-34 % (Figure 2A). Metabolites of isolate KT.16 were capable of inhibiting mycelial growth of isolate FB.2 up to 32.67 %, then followed by isolate KT.7 and KT.10 (Figure 2B). There were 18.27 % and 15.54 % respectively. Maximum inhibition of isolate FB.1 by isolate KT.16 had been shown in antagonist assay. This result indicated that this isolate was prime antagonist mold with potential metabolites and ability to compete with pathogenic *Fusarium*.



A B

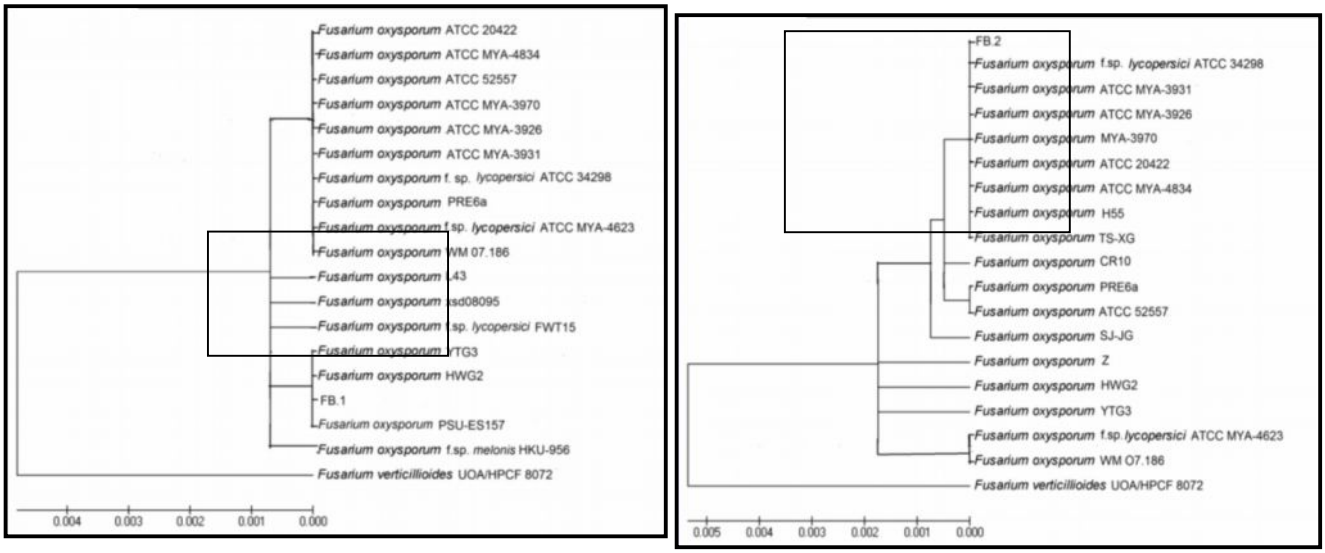
Figure 2. Inhibition percentage of *Fusarium* by non-volatile metabolite of antagonist molds

Different results can be affected by different production phase of metabolites. Type of metabolites is also important to be considered. The low inhibition percentage might be caused by lower concentration of metabolites. Culture filtrate of *T. longibrachiatum* at concentration 25 % reduced colony diameter of *F. oxysporum* f. sp. *lycopersici* 78.76 % after 7 days incubation time[27]. Different concentrations of culture filtrate from *Trichoderma* were effective for controlling *F. oxysporum* f. sp *lycopersici* and *F. solani* causing wilt disease on tomato plants. Combination of secondary metabolites and lytic enzyme resulted synergy effect in controlling growth of *F. oxysporum*[26].

Isolate KT.7 known as *Aspergillus niger* showed higher inhibition percentage than isolate KT.10 against *Fusarium* FB.1 and FB.2. According to Enespa and Dwivedi (2013) study, culture filtrate of *A. niger* with lower concentration (25 %) showed inhibition activity (81 %) against *F. oxysporum* f.sp. *lycopersici*, higher than *T. koningii* dan *T. viride*[28]. It has been reported that *A. niger*, *A. fumigatus*, and *A. flavus* produce secondary metabolites, such as peptide, alkaloid, tunicamycin, austinol, and gliotoxin[29]. One of the non volatile metabolites of molds was peptaibol. This compound had synergy effect with β-glucanase and inhibited cell wall synthesis of pathogen by preventing synthesis of β-glucan synthase[30]. Trichogins A from *T. longibrachiatum* and *tricodecenins* from *T. viride* were lipopeptaibol compounds with antifungal activity. Another metabolite was *Trichothecenes harzianum* A which activated gene expression in plant resistance system on tomato plants[31].

Identification of *Fusarium* and antagonist mold

DNA sample of antagonist mold and *Fusarium* produced amplicon 600 and 700 bp. These results are similarly with Lian et al (2008) who observed DNA amplification using primer ITS4 and ITS5 yielded DNA fragment of approximately 600-700 bp[32]. Based on *alignment* results of ITS region, isolate FB.1 had similarity 100 % with *F. oxysporum* YTG3, HWG2, PSU-ES157, *F. oxysporum* f.sp *lycopersici,* and *F. oxysporum* f.sp. *melonis* (Fig. 3A). The phylogeny tree was rooted from *F. verticillioides* as outgroup. Phylogeny tree showed that isolate FB.2 had similarity value 100 % with *F. oxysporum* f. sp. *lycopersici* ATCC 34298 (Fig. 3B). ITS region was used as DNA barcode for identification fungi since it occurs in multiple copies, conserved, and high degree of variability sequences[33.] Similarity value 89 to 98 % indicated isolates were included in one genus. Similarity value over 99% identified as spesies, while 100 % classified as same strain[17]. Isolat KT.16 was *T. longibrachiatum* ATCC 52326 since it had similarity value 100 % with reference isolates (Fig. 4). The member of *Trichoderma section longibrachiatum* are *T. citrinoviride*, *T. atroviride*, *T. pseudokoningii*, and *T. longibrachiatum*[34]. Potency of *T. longibrachiatum* as biological control agent had been reported. This mold can be used to control pathogen growth, such as *F. oxysporum, F. moniliforme*, and *F. solani* [20].



A B

Figure 3. Phylogeny tree of *Fusarium* isolates with reference isolates based on Maximum Likelihood algorithm

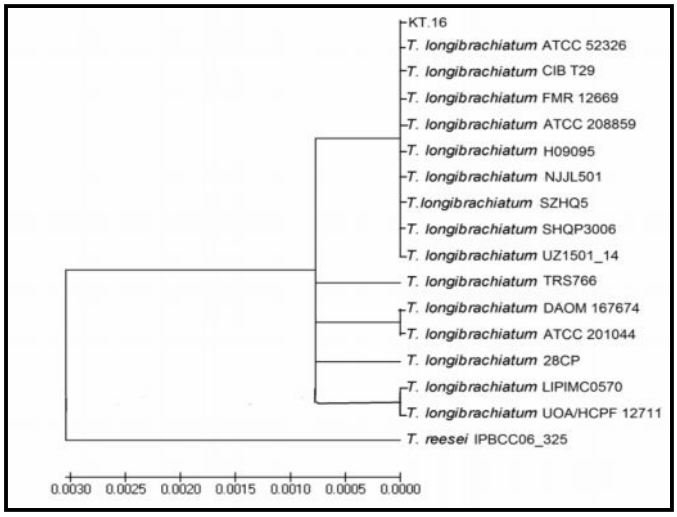


Figure 4. Phylogeny tree of isolate KT.16 with reference isolates based on Maximum Likelihood algorithm

Conclusion

Isolate KT.16 had antagonist activity against *Fusarium* isolate FB.1 (59.84 %), whereas isolate KT.10 against *Fusarium* isolate FB.2 (54.67 %). Non-volatile metabolites of isolate KT.16 showed the highest inhibition percentage against both *Fusarium*. Isolate KT.16 was the best antagonist mold and was identified as *Trichoderma longibrachiatum* ATCC 52326 with similarity 100 %.

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