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Identification of antagonistic fungi from apple in Batu City, Indonesia

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Abstract

Pathogenic fungi are organisms that causes diseases and death on plant. Biological control for pathogenic fungi can be done by applying antagonistic fungi. The aims of this study are identify the three isolates of antagonistic fungi (T1, T6 and A2) and support the effort of control of pathogenic fungi and biofungicide production. The methods in this study included monospore subculture of pathogenic fungi and antagonistic fungi, DNA isolation, amplification using ITS5/ITS4 primer, purification, sequencing and sequence homology analysis of each isolates. The result showed that T1 and T6 had similarity value of DNA sequence 90.96 % and similarity value between those isolates with A2 was less than 53 %. The result also showed that antagonistic fungi T1 was determined as *Trichoderma asperellum* with similarity value 100 %, T6 was determined as *Trichoderma harzianum* with similarity value 100 %, and A2 had relationship with *Aspergillus flavus* with similarity value 100 %. Based on the result, so we can conclude that the two isolates (T1 and T6) were same as one Genus, *Trichoderma,* while isolate A2 was belong to into Genus *Aspergillus*.

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Introduction

Apple crop is one of horticultural plant with high value in Indonesia. Apple crops were introduced by Europeans during colonization and naturalized become tropical apple. One of the agricultural center of apple in Indonesia is East Java, especially in Malang, Batu City and surrounding areas. However, decrease in the domestic apple productivity in some agricultural centers of apple, especially in Batu City causing apple demand fulfillment is not achieve. One cause of the decline in productivity of it is pathogenic fungi attack (Hakim and Siswanto, 2009). Pathogenic fungi affecting apple crop productivity decline by 90 % (Balitjestro, 2011).

Pathogenic fungi that attack apple crops in Batu City include in Genus *Pythium*, Spesies *Pythium splendens* (Nugrahani *et al.,* 2012; Lestari *et al.,* 2013). *Pythium* was infected in a wide range of crops, seeds and death of plant. It also cause decreased ability of root to get sources of nutrients from soil significantly (Linde *et al.*, 1994; Ploetz, 2005; Teymoori *et al.*, 2012). In addition, it was being one of the causes of diseases in apple crops (such as custard apple and sugar apple). Beside that, *Pythium* also cause disease in numbers of other crops, for example atemoya, cherimoya in California and Florida, cucumbers, bananas, taro, palm, soybean, rice and ornamental crops, for example *Begonia* sp., *Coleus* sp., *Epipremnumaureum*, *Peperomia caperata*, *Philodendron scandens* subsp. *Oxycardium*, *Chrysanthemum* spp., *Cymbidium* spp., *Pelargonium* spp., etc. (Linde *et al.,* 1994; Ploetz, 2005; Ho *et al.*, 2012; Van Buyten *et al.*, 2013).

Handling pathogenic fungi attacks have generally been carried out with the sanitation system settings and systemic fungicide spraying. Systemic fungicide work by going into the vascular system of plants and inhibit infection by pathogenic fungi that attack plant tissues. Application of systemic fungicides such as metalaxyl (Subdue 2E), ethazol (Truban), mefenoxam, azoxystrobin, trifloxystrobin, and captan are known to inhibit pathogenic fungi such as *Pythium* if the light level attacks (Uchida, 1990; Broders *et al.*, 2007). However, the use of systemic fungicides continuously at a dose that does not conform feared could trigger the emergence of new strains of pathogenic fungi and cause environmental pollution and mortality to other organisms in the surrounding environment. Therefore, it is necessary to biological agents that can inhibit the infection of pathogenic fungi (Uchida, 1990; El-Katatny *et al.*, 2004; Broders *et al.*, 2007; Hardaningsih, 2011).

Several antagonistic fungi obtained from Nugrahani *et al.* (2012) that isolated from soil around apple plant were infected by pathogenic fungi. Some antagonistic fungi (T1, T6 and A2) have different ability to inhibit of pathogenic fungi growth. Inhibitory percentage of antagonistic fungi reached 47.94 % - 62.95 %. Mechanism of antagonistics fungi to inhibit of pathogenic fungi growth for example throughout microparasitism and antibiosis with produce secondary metabolites (Alabouvette *et al.* 2006; Nugrahani *et al.*, 2012).

Antagonistic fungi in earlier study were also identified using phenotype analysis (Nugrahani *et al.*, 2012). The result showed that antagonistic fungi isolates, T1 and T6 were suspected in Genus *Trichoderma* and A2 were suspected in Genus *Aspergillus*. Phenotypic identification was difficult and ineffective because some phenotypic characters were able to overlap between a species with another species and possible occurrence of each isolates changes (Ristaino *et al.*, 1998; Hardaningsih, 2011). Identification of species was defined on the basis of phenotypic characters and genotype. Each species have unique phenotypic characters. The combination of phenotype and genotype was required to support taxonomy and classification of species (Staley, 2009). Therefore, identify the three isolates of antagonistic fungi (T1, T6 and A2) using molecular analysis in this study was required to support phynotype analysis.

Materials and methods

The Source of antagonistic fungi isolate

Antagonistic fungi isolates were obtained from Nugrahani research (Nugrahani *et al.*, 2012). It was isolated from soil samples under infected apple plants canopy in five different sides.

DNA isolation of fungi isolate

Three isolates of fungi were subcultured in 25 ml of PDB (Potato Dextrose Broth) medium then incubated in a shaker incubator at 28°C,120 rpm for three days. DNA genome was isolated from the mass of fungi mycelium after incubated for three days using a modified method with different lysis buffer (Tris-Cl, pH 8, EDTA 0.5 M, pH 8, SDS 10%, ddH_2O) (Doyle and Doyle, 1990).

Polimerase Chain Reaction (PCR) *of fungi isolates*

ITS region was amplified using ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The DNA sequence amplification reaction condition are shown in Table 1. The primers ITS5 and ITS4 were produced a single band of approximately 900 bp (Cooke *et al.*, 2000). The PCR result were visualized by electrophoresis and documented by UV transluminator.

DNA sequencing of fungi isolates

Purificarion and Sequencing of 18S rDNA sequence performed in Macrogen, South Korea.

Phylogenetic analysis

Data sequences of antagonistic fungi isolates, several isolates of the reference sequence (*Trichoderma* and *Aspergillus*) from the GeneBank (http://www.ncbi.nlm.nih.gov) were presented in FASTA format. Analysis of DNA sequences and construction of phylogeny tree were done using MEGA ver. 5.03 program program with 1000 bootstrap, inversion using Maximum Likelihood methods and evolutionary distance were analyzed according to Tamura- Nei model (Tamura and Kumar, 2002).

Result and discussion

In earlier study (Nugrahani et al., 2012) research antagonistic fungi isolates showed that T1 and T6 were suspected in Genus *Trichoderma* and A2 was suspected in Genus *Aspergillus* (Fig. 1). In this study, morphological data will examine and compare with molecular data for identify those isolates.

Table 1. Reaction condition for PCR (Cooke *et al.*, 2000; Suharjono *et al.*, 2010; Schoch *et al.* 2011).

No.	Reaction	Temperature (^{0}C)	Time (min)
1	Pre Denaturation	95	10
	35 cycle:		
2	Denaturation	94	0,25
	Annealing	53	0,5
	Extension	72	0,25
3	Post extension	72	7

Dendogram analysis of morphological data showed that antagonistic fungi isolates, T1 had similarity value of 86.84 % with T6 isolates and A2 antagonist fungi isolates had similarity value of 50.88 % with T1 and T6 isolates. In addition, similarity value of T1 and T6 isolates with *Trichoderma* was more than 85 %, whereas A2 isolates showed similaritas value of 100% with *Aspergillus* (Fig. 1). Species concept of morphological character, if species had similarity value more than 50 % with other species, the isolates were grouped in one Genus (Priest and Austin, 1993; Staley, 2009).

Based on species concept, three isolates of antagonistic fungi (T1, T6 and A2) were included in one Genus with similarity value more than 50 %.

The result showed that morphological characters were not enough for distinguishing those isolates. However, those isolates were grouped in different Genus (Fig. 2). Identification of species using morphological character need a long time and specific characters, have high percentage of error and effect of morphological similarity (Siricord, 2005; Villa *et al.*, 2006). Overall, molecular studies of those isolates have supported identification of it.

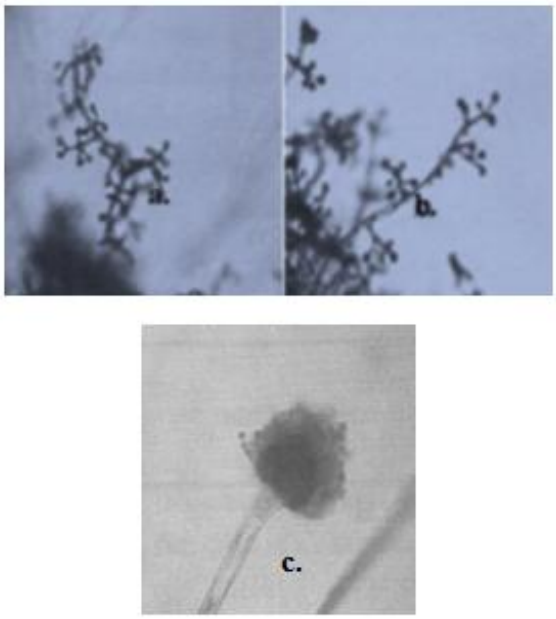

Fig. 1. Morphological character of antagonistic fungi. a. T1, b. T6 dan c. A2 (Nugrahani *et al.*, 2012).

The results of DNA amplification of antagonistic fungi isolates using ITS sequence with ITS5/ITS4 produced DNA amplicon approximately 900 bp (Fig. 3). DNA sequence of antagonistic fungi isolates were aligned with reference isolates (*Trichoderma* and *Aspergillus*). The DNA sequence showed that sites with alignment gaps of 112 nucleotides, invariable (monomorphic) site of 233 nucleotides and variable (polymorphic) sites of 227 nucleotides. The result also showed that there were total numbers of mutations in variable site of 308 nucleotides with singleton variable sites of 38 nucleotide and parsimony informative sites of 189 nucleotides.

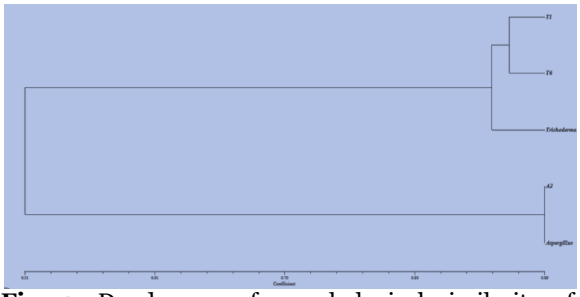

Fig. 2. Dendogram of morphological similarity of antagonistic fungi isolates and reference isolates.

Topology of tree and bootstrap analysis of DNA sequences indicated that antagonistic fungi isolates (T1, T6 and A2) include in different Genus (Fig. 4). The phylogenetic analysis also showed that T1 isolate had similarity value of 90.96 % and evolutionary distance of 0.090 with T6 isolate and those isolates (T1 and T6) had similarity value less than 53 % with A2 isolate. However, the results also indicated that T1 isolate is related more closely to *Trichoderma asperellum* Tr48 with similarity value of DNA sequence of 100 % and T6 isolate is more closely related to *Trichoderma harzianum NR6929* and *Trichoderma harzianum* ATCC 20847 with similarity value of 100 %. In addition, A2 isolates had similarity value of 100 % with reference isolates of *Aspergillus flavus* C1931 and *Aspergillus flavus* C8467 (Fig. 4).

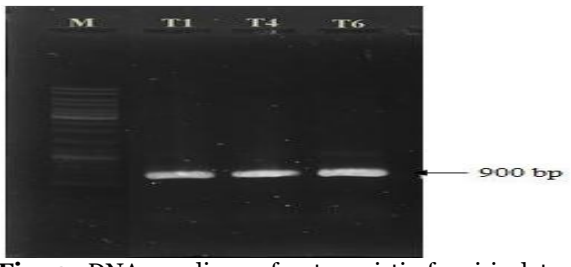

Fig. 3. DNA amplicon of antagonistic fungi isolates (T1, T6 and A2) with a marker (GeneRuler™ DNA Ladder Mix 100).

Phylogenetic analysis of DNA sequence was not determined of specifically antagonistic fungi strain. Species concept of phylogenetic analysis, if species had similarity value of 89-99 % included in similar Genus and similarity value more than 99 % included in similar species and also similarity value of 100 % indicated that species in similar strain (Henry *et al.*, 2000 cit . Suharjono *et al.*, 2010).

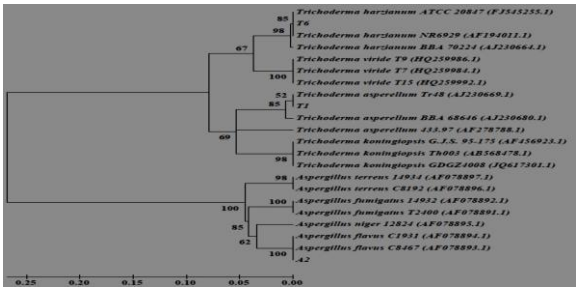

Fig. 4. Phylogeny tree of antagonistic fungi and reference isolates (Trichoderma and *Aspergillus*) based on Maximum Likelihood alogaritm with Tamura-Nei method.

DNA sequence of ITS region using ITS5 and ITS4 primer were expected to amplify partial sequence of 18S rDNA and 28S rDNA, complete sequence of 5.8S rDNA, ITS1 and ITS2 (universal DNA barcode marker of fungi) (White *et al*., 1990; Schoch *et al*. 2012). ITS region had barcode gap analysis of interspecific value of 0.372664, it was higher than other marker (SSU, LSU and RPB1 marker), but intraspecific value of it was not enough determine species in intra-species than other marker (Schoch *et al*. 2012). The result showed that ITS region can determine species in inter-species more effective than other marker, but it was not enough to determine species in intra-species.

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