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Antagonistic activity of three *Aspergillus* isolates against *Fusarium* wilt of banana

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Abstract

Endophytic fungi have been known to play a role in protecting their host plants against diseases and extreme environments. The purpose of this study was to determine the antagonistic activity of three *Aspergillus* isolates against *F. oxysporum* f. sp. *cubense* (*Foc*), and to identify the *Aspergillus* identity using multilocus phylogeny analysis. Antagonistic assay by dual culture method showed that the *Aspergillus* sp. strain PD2, strain PD4, and strain PD5 inhibited the growth of *Foc* isolate by 37.31%, 26.52%, and 12.04%, respectively. Multilocus phylogeny based on ITS rDNA, beta-tubulin, and calmodulin genes sequences showed that the *Aspergillus* strain PD2 and strain PD4 belong to *A.* section *Terrei*, while the *Aspergillus* sp. strain PD5 was identified as *A. sydowii* of the *A.* sect. *Versicolores*.

Keywords: biocontrol, endophyte, fungi, *Fusarium* wilt, multilocus phylogeny

Introduction

One of the most destructive diseases of banana and difficult to overcome is a *Fusarium* wilt disease caused by *F. oxysporum* var. *cubense* (*Foc*) (Simmonds 1966), specifically tropical race 4 (TR4) (Ploetz 2006). *Foc* TR4 was first discovered in the Southeast Asia region and continues to spread to Africa, West Asia, East Asia, and Australia (Ploetz 2015a). In Indonesia, *Foc* TR4 was reported to be spread in Bali, Halmahera, Kalimantan, Java, Papua, Sulawesi, and Sumatra (Ploetz 2015a).

Various methods for controlling *Foc* TR4 have been widely reported. This includes chemical controls such as the use of benzimidazole fungicides, demethylation inhibitors, phosphonate, and strobilurin (Nel *et al*. 2007), cultural measures through heating applications for soil sterilization, addition of nitrate (NO3) and ammonia (NH4) into soil, application of disease suppressive soil (Peng *et al*. 1999, Molina *et al*. 2010, Elmer 2012), biological method through the use of biocontrol microorganisms (Fravel *et al*. 2003), and the use of cultivars that are resistant to *Foc* TR4 (Xu *et al*. 2011). To date, applicable control methods of *Foc* TR4 on a large scale and showing promising results are biological control methods and the use of cultivars that are resistant to *Foc* TR4 (Ploetz 2015b).

Among taxa that are potentially used as biocontrol agents, members of the genus *Aspergillus* such as *A. flavus*, *A. flavipes*, and *A. niger* were reported to be potentially developed as biocontrol agents (Tiwari *et al*. 2011, Wang *et al*. 2014). In this study, three endophytic fungal isolates that morphologically belong to the genus *Aspergillus* were tested

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for their antagonistic activity against *Foc*. These fungal isolates were further identified using multilocus phylogenetic analysis based on ITS rDNA, beta tubulin, and calmodulin genes.

Materials and Methods

Microorganisms

All fungal isolates used in this study were obtained from Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Jakarta (UNJ).

Antagonistic assay by dual culture method

Interaction between antagonistic fungi (*Aspergillus* spp. strain PD2, PD4, and PD5) and pathogenic fungus (*F. oxysporum* f. sp. *cubense*) were determined by dual culture method described by Dennis & Webster (1971). In a sterile condition, mycelium of seven days *Aspergillus* isolate from Oatmeal Agar (OA) medium was picked out using inoculation loop and was placed on right edge of petri dish containing Potato Dextrose Agar (PDA) medium, and the mycelium of *Foc* was placed on left edge of the same petri dish. The distance between fungal isolates was 3 cm. The plates were incubated at room temperature and observed after seven days.

The diameter of *Foc* colony in the antagonistic test and in the negative control was measured. The growth inhibition was calculated using the following formula (Skidmore & Dickinson 1976):

$$P = \frac{(C - T)}{T}$$

P = Inhibition activity (%)
C = Diameter of *Foc* colony in negative control (cm)
T = Diameter of *Foc* colony in the antagonistic assay (cm)

Growth inhibition of *Foc* was analyzed by ANOVA. If it shows significant results, it was followed by Duncan Multiple Range Test (DMRT) 5% to find out if there was a real difference in the growth of *Foc* colony caused by the antagonistic fungal isolate.

Multilocus phylogenetic analysis

DNA extraction, PCR amplification and sequencing

Seven days mycelium of fungal isolates were extracted using Ilustra Nucleon™ Phytopure™ Genomic DNA Extraction Kit (GE Healthcare) according to the manufacturer's protocol. PCR amplification was conducted using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al.* 1990) to amplify the ITS rDNA region, Bt2a (5'-GGT AAC CAA ATC GGT GCT GCT TTC-3') and Bt2b (5'-GGT AAC CAA ATC GGT GCT GCT TTC-3') (Glass & Donaldson 1995) to amplify beta-tubulin gene, and Cmd5F (5'-CCG AGT ACA AGG ARG CCT TC-3') and Cmd6R (5'-CCG ATR GAG GTC ATR ACG TGG-3') (Hong *et al.* 2005) to amplify calmodulin gene. PCR mixture was composed of 12.5 µL GoTaq® Green Mastermix (Promega, USA), 10 µL nuclease free water (NFW), 0.5 µL forward dan reverse primer, 0.5 µL DMSO, and 1 µL DNA template.

PCR reaction for the ITS region was set as follow: pre-denaturation at 95ºC for 3 min, followed by 35 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, elongation at 72ºC for 1 min, and final elongation at 72ºC for 7 min. For beta-tubulin and calmodulin amplifications, annealing temperature was set at 58ºC for 1 min (Hageskal *et al*. 2011,

Howard *et al*. 2011). All DNA amplifications were carried out using T100™ Thermal Cycler (BioRad, USA). The PCR products were electrophorized using 1% agarose gel at a 100 V for 20 min. 1 Kb DNA ladder was used as a marker. The agarose gel was further soaked in a GelRed for 15 min and visualized using Gel Doc™ EZ Gel Documentation System (BioRad, USA). The PCR products were sent to First Base (Malaysia) for sequencing.

Phylogenetic analysis

Newly nucleotide sequences from the ITS1-ITS4, Bt2a-Bt2b, and Cmd5F-Cmd6R primer pairs were examined and assembled using ChromasPro version 1.7.7 (Technelysium Pty Ltd, Australia). Homologous sequences of these sequences were searched using BLAST (Basic Local Alignment Search Tool Algorithm) (Altschul *et al*. 1997) in the NCBI GenBank database (www.ncbi.nlm.nih.gov). All GenBank accession codes of the sequences used in this study were listed in the table 1. Phylogenetic tree from a single gene or region was reconstructed using Neighbour Joining (NJ) method implemented in MEGA (Molecular Evolutionary Genetic Analysis) version 7.0. (Kumar *et al*. 2016). Multilocus phylogenetic analysis from the ITS rDNA, beta tubulin, and calmodulin genes was conducted using Maximum Parsimony (MP) method implemented in PAUP* (Phylogenetic Analysis Using Parsimony and other methods) 4.0b10 (Swofford 2002). The heuristic search option was using the 'tree–bisection–reconstruction' (TBR) algorithm with 1000 random sequence additions to find the optimum tree. The stepwise addition option set as random and maximum tree number was set at 500. Tree length (TL), consistency index (CI), retention index (RI), related consistency index (RC), and homoplasy index (HI) were also calculated. The strength of the internal branches of the phylogenetic tree in MP analysis was tested with bootstrap (BS) analysis using 1000 replications. BS values of 50% or higher than that are shown and gaps were treated as missing data. The partition homogeneity test (Farris *et al*. 1995) with 1000 replicates, 10 random addition sequence replicates, and TBR branch swapping was conducted by using PAUP* to determine whether ITS, beta-tubulin and calmodulin datasets were in conflict. A significance level of P = 0.01 was adopted for this test (Cunningham 1997).

Table 1. List of nucleotide sequences used in this study

Section	Spesies	GenBank Accession number		
		ITS	**Beta-tubulin**	**Ccalmodulin**
Terrei	*A. neoafricanus*	NR135331	EF669516	EF669543
	A. alabamensis	NR135428	EU147769	EU147583
	A. allahabadii	NR135399	EF669531	EF669559
	A. ambiguus	NR135400	EF669534	EF669564
	A. aureoterreus	EF669580	EF669524	EF669538
	A. carneus	EF669611	EF669529	FJ531220
	A. floccosus	FJ531205	FJ491714	FJ531219
	A. hortai	FJ531192	FJ491706	FJ531242
	A. microcysticus	EF669607	EF669515	EF669565
	A. neoindicus	EF669616	EF669532	EF669574

Section	Spesies	GenBank Accession number		
		ITS	**Beta-tubulin**	**Ccalmodulin**
	A. neoniveus	NR_137474	EU014098	EF669570
	A. niveus	NR137476	EF669528	EF669573
	A. pseudoterreus	NR137472	EF669523	EF669556
	A. terreus	EF669586	EF669519	EF669544
Versicolores	*A. creber*	NR_135442	JN853980	JN854043
	A. puulaauensis	NR_135445	JN853979	JN85403
	A. tennesseensis	NR_135447	JN853976	JN854017
	A. cvjetkovicii	EF652440	EF652264	EF652352
	A. jensenii	NR_135444	JN854007	JN854046
	A. venenatus	NR_135448	JN854003	JN854014
	A. sydowii	EF652450	JN853933	EF652362
	A. versicolor	EF652442	JN853941	EF652354
	A. fructus	EF652449	JN853942	EF652361
	A. tabacinus	NR_135361	JN853945	EF652390
	A. amoenus	NR_137462	JN853946	EF652392
	A. austroafricanus	NR_135443	JN853963	JN854025
	A. protuberus	NR_135353	JN853964	EF652372
	A. subversicolor	NR_135446	JN853970	JN854010
Flavi	*A. flavus*	AF027863	EF661508	KJ175479

Results

Dual culture antagonistic assay

The antagonism assay showed the formation of an inhibition zone between fungal antagonistic agents (*Aspergillus* sp. strain PD2, *Aspergillus* sp. strain PD4, *Aspergillus* sp. strain PD5) and pathogenic fungus (*F. oxysporum* f. sp. *cubense*) (Fig. 1). The form of *Foc* colony was imperfect because the part of this pathogenic fungus colony that facing *Aspergillus* colony did not grow well due to inhibition by the *Aspergillus* colony.

Duncan multiple range test (DMRT) of the antagonistic assay of the *Aspergillus* spp. isolates (strain PD2, strain PD4, and strain PD5) showed that there was a difference in the inhibition activity of these *Aspergillus* spp. isolates on the growth of *Foc*. The highest growth inhibition of *Foc* exhibited by *Aspergillus* sp. strain PD2 (37.31%), followed by *Aspergillus* sp. strain PD4 (26.52%), and *Aspergillus* sp. strain PD5 (12.04%) (Fig. 2).

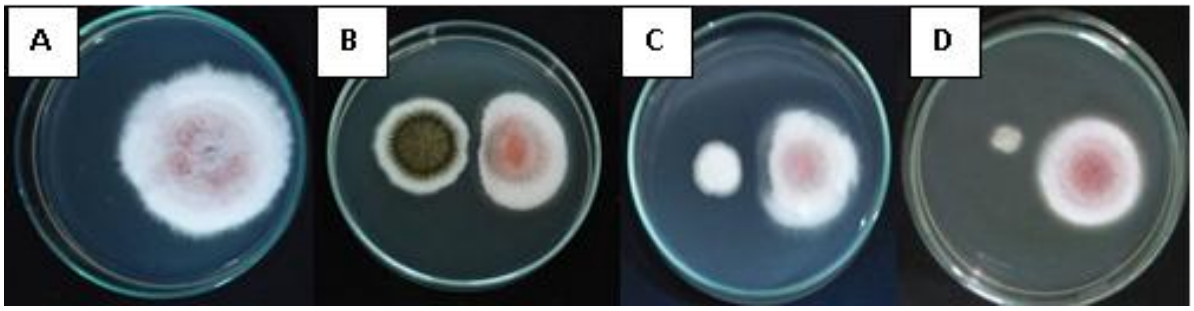

Figure 1. Dual culture method of antagonistic assay after 7-d incubation on PDA medium. (A) *Foc* isolate, (B) *Aspergillus* sp. strain PD2 against *Foc*, (C) *Aspergillus* sp. strain PD4 against *Foc*, (D) *Aspergillus* sp. strain PD5 against *Foc*.

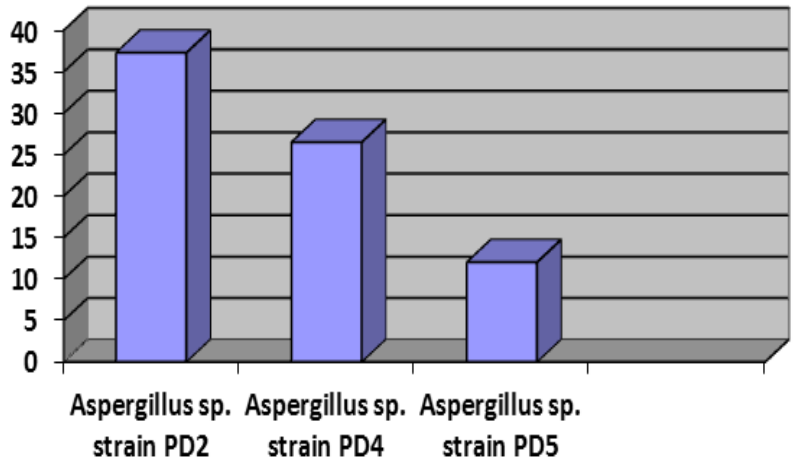

Figure 2. Percent growth inhibition of *Foc* isolate by *Aspergillus* spp. Isolates after 5-d incubation at room temperature

Multilocus phylogenetic analysis

The BLAST results of the ITS sequence of *Aspergillus* sp. strain PD2 and strain PD4 indicated a close genetic relationship between these sequences with the *Aspergillus* sequences that belong to the *A.* section *Terrei* (data not shown). Therefore, we reconstructed a multilocus phylogenetic tree of the *Aspergillus* sp. strain PD2 and strain PD4 with the type sequences of the *Aspergillus* that belong to the sect. *Terrei*. The multilocus phylogenetic tree generated from the ITS rDNA region, beta-tubulin, and calmodulin genes showed that *Aspergillus* sp. strain PD2 and strain PD4 sequences nested in the same clade with *A. neoafricanus* strain NRRL 2399, *A. alabamensis* strain UAB 20, *A. hortai* strain NRRL 274, dan *A. terreus* strain NRRL 255 with 74% bootstrap support (BS) (Fig. 3). Additional sequences from another gene or region is necessary to determine the species name of *Aspergillus* sp. strain PD2 and strain PD4.

In addition, the BLAST results of the ITS sequence of *Aspergillus* sp. strain PD5 indicated that this sequence belong to the *Aspergillus* sect. *Versicolores* (data not shown). The multilocus phylogenetic tree showed that *Aspergillus* sp. strain PD5 sequence formed a monophyletic clade with *A. sydowii* strain NRRL 250 with 100% BS (Fig. 4). Therefore, *Aspergillus* sp. strain PD5 was determined as *A. sydowii*.

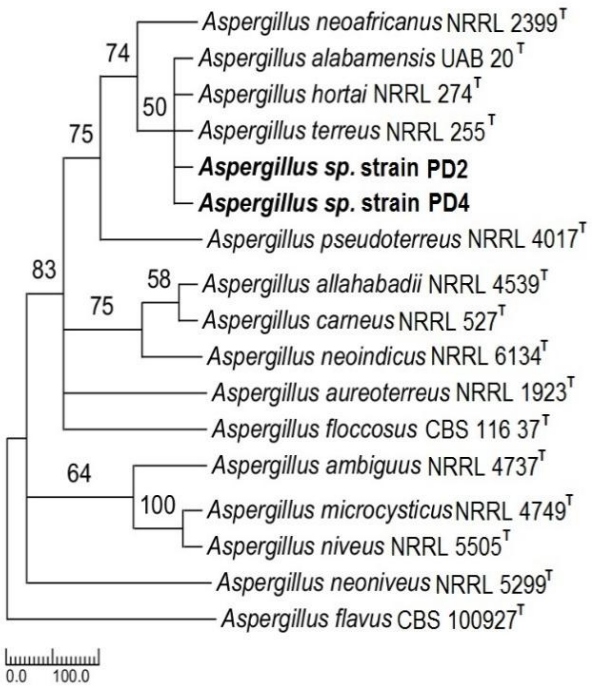

Figure 3. Phylogenetic tree generated from the MP analysis of the ITS rDNA region, beta-tubulin, and calmodulin genes combined sequences showed a relationship between *Aspergillus* sp. strain PD2 and strain PD4 with closely related species from *A.* sect. *Terrei*. Bootstrap value from 1000 replicates was showed above the branch nodes.

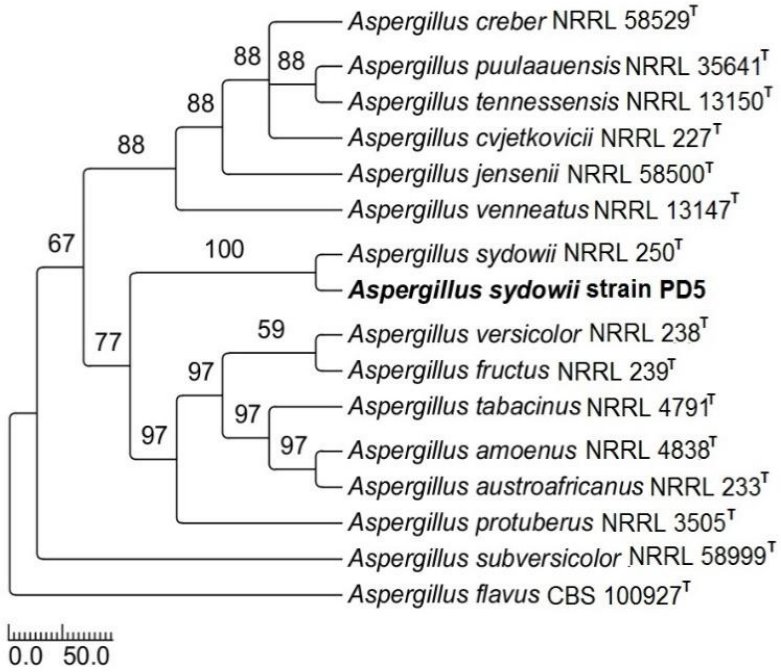

Figure 4. Phylogenetic tree generated from the MP analysis of the ITS rDNA region, beta-tubulin, and calmodulin genes combined sequences showed a relationship between *Aspergillus* sp. strain PD5 with closely related species from *A.* sect. *Versicolores*. Bootstrap value from 1000 replicates was showed above the branch nodes.

Discussion

The antagonistic assay showed that *Aspergillus* sp. strain PD2, *Aspergillus* sp. strain PD4, *A. sydowii* strain PD5 could inhibit the growth of *Foc* isolate (Figs. 1-2). It was shown by the formation of the inhibition zone between the colonies of *Aspergillus* and *Foc* isolates in the antagonistic assay. The inhibition zone and imperfect colony shape are clear indicators of bioactive compound secretion that inhibit the growth of fungal mycelia through lysis of fungal cell wall (Gomathi & Ambikapathy 2011). Several studies have shown that members of the genus *Aspergillus* produce lytic enzymes such as chitinase (Farag *et al*. 2016), glucanase (Gao *et al*. 2008), and proteases (Sethi *et al*. 2016). In addition. members of the *Aspergillus* have been known for their capacity in producing bioactive compounds such as *A. niger* (Tiwari *et al*. 2011) and *A. terreus* (Goutam *et al*. 2017). ‘’Terrein’’ is an antimicrobial and antitumor compound produced by *A. terreus* (Goutam *et al*. 2017).

In addition, many *A.* sect. *Terrei* members have also been known for their potential in producing secondary metabolites, such as *A. terreus* that produced ‘’Terrein’’ (Goutam *et al*. 2017), antimicrobial methyl 3,4,5-trimethoxy-2-(2-(nicotinamido) benzamido) benzoate (Wang *et al*. 2011), and another antimicrobial compound against several fish pathogens (*Edwardsiella tarda*, *Aeromonas hydrophila*, *Vibrio ordalii*, and *V. anguillarum*) (Barakat & Gohar 2012), *A. alabamensis* that produced two diketomorpholine derivatives and a highly conjugated ergostane-type steroid that exhibited inhibitions against human pathogens (*E. coli* and *M. luteus*) and aquatic bacteria (*E. ictaluri* and *V. alginolyticus*) (Yang *et al*. 2018), *A. allahabadii* that produced antibacterial allahabadolactone B (2) and (22E)-5α,8α-epidioxyergosta-6,22-dien-3β-ol against *B. cereus* (Sadorn *et al*. 2016), and pyrone derivatives: 3-hydroxy 2-methyl 4-pyrone and 5-hydroxy-2-(hydroxymethyl)-4H-pyrone that showed potent antimicrobial, antioxidant, antidiabetic, and mosquito larvicidal activities (Rajamanikyam *et al*. 2017), *A. carneus* produced a potential antimicrobial compounds prenylated indole alkaloids, carneamides A-C (1–3), quinazolinone derivatives, carnequinazolines A-C (5–7), aryl C-glycosides, carnemycin A, B (8, 9), and a drimane sesquiterpenoid (Zhuravleva *et al*. 2012), *A. niveus* that produced a high antitumor, but weak antibacterial Aspochalamins A-D (Gebhardt *et al*. 2004), *A. microcysticus* that produced a well-known antimicrobial compound, Asposterol (Heberle *et al*. 1974).

Aspergillus sydowii, another *Aspergillus* species determined in this study, has been known as saprobe in soil as well as pathogen to human (Chiu *et al*. 2005), animal (Lee *et al*. 2012), and corals (Greco *et al*. 2017). Although many strains of *A. sydowii* causes disease in several groups of organisms, however, several strains of *A. sydowii* were reported to produce potential antimicrobial bioactive compounds such as a bisabolane-type sesquiterpenoids, namely aspergillusene D, two new xanthones, and two new catechol-derivatives, of which the xanthones displayed selective inhibitory activities against two influenza A virus (Liu *et al*. 2019), and sydowiols A–C (1–3) that exhibited protein tyrosine phosphatase inhibitors activity of *Mycobacterium tuberculosis* which is important for the treatment of tuberculosis (Liu *et al*. 2013).

Conclusion

Aspergillus sp. strain PD2, *Aspergillus* sp. strain PD4, *A. sydowii* strain PD5 showed in vitro antagonistic activity against *F. oxysporum* f. sp. *cubense*. Additional sequence data from RNA polymerase II second largest subunit (RPB2) gene is necessary to determine the identity of *Aspergillus* sp. strain PD2 and *Aspergillus* sp. strain PD4.

Conflict of Interest

The authors state no conflict of interest from this manuscript.

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