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...
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 TCC-3’) and Bt2b (5’-GGT AAC CAA ATC GGT GCT GCT TCC TTC-3’) (Glass & Donaldson 1995) to amplify beta-tubulin gene, and Cmd5F (5’-CCG AGT ACA AGG ARG CCT TC-3’) and Cmd6F (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 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min, and final elongation at 72°C for 7 min. For beta-tubulin and calmodulin amplifications, annealing temperature was set at 58°C 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).

| Section | Spesies             | GenBank Accession number |   |
|---------|---------------------|--------------------------|---|
| Terrei  | A. neoaficanus      | 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 | Calmodulin |
|----------|-----------------------------|--------------------------|--------------|--------------|------------|
|          | 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. paulaaensis              | 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, *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 (2E)-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), quinazolione 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. micocysticus* that produced a well-known antimicrobial compound, Aspsterol (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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