Endobacterial symbiont of *Fusarium oxysporum* f.sp. *cubense* and the pathogenicity of their symbiosis towards banana plantling

G Rahayu¹*, I Maulana¹ and Widodo²

¹Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Jl Agatis Kampus IPB Dramaga, Bogor, 16680, Indonesia
²Department of Plant Protection, Faculty of Agriculture, IPB University, Jl Agatis Kampus IPB Dramaga, Bogor, 16680, Indonesia

*Corresponding author: gayuhrahayu@gmail.com

Abstract. *Fusarium oxysporum* f.sp. *cubense* (Foc), a causal agent of panama disease was reported carrying bacterial endosymbionts. This research aimed to identify the endobacteria in 7 Foc strains and to study their symbiosis effect on the Foc pathogenicity towards Ambon Kuning and Tanduk banana cultivars. The pathogenicity of endobacteria-Foc symbiosis were tested in vitro on banana plantling. Crude filtrate and biomass suspension that were produced by culturing each Foc strains for 21 days in static condition either in PDB (Potato Dextrose Broth) (Foc⁻) or in antibiotics supplemented PDB (Foc⁺) were used as effectors. The Foc pathogenicity was stated as disease severity percentage on leaves and on rhizome. The result showed that all Foc strains contained endobacteria. *Enterobacter* sp. was hosted by Foc IPBCC 19.1427 and *Izhakiella australiensis* was in the hyphae of Foc IPBCC 19.1430. *Enterobacter* sp. affected Foc IPBCC 19.1427 virulence towards banana cv. Tanduk through effectors in the filtrate, while that towards cv. Ambon Kuning was unclear. *Izhakiella australiensis* did not affect Foc IPBCC 19.1430 virulence towards the two banana cultivars. The pathogenicity of Foc should further be verified, as the antibiotics given could not completely kill the endobacteria.

Keywords: 16S rDNA, endosymbiosis, Panama disease, pathogenicity

1. Introduction

Living fungal hyphae have been reported to harbour some bacteria [1-8]. Bacteria such as *Acinetobacter junii*, *Bacillus atrophaeus*, *B. foraminis*, *B. subtilis*, *Oxalobacteraceae* sp., *Pantoea agglomerans*, *Paenibacillus* sp., and *Variovorax paradoxus* were found to be the endobacterial symbionts of endophytic Ascomycota (Eurotiomycetes, Dothideomycetes and Sordariomycetes) [1]. Uptill now, it is found that fungal microbiome consists of one or more endobacteria. *Gigaspora margarita* carries *Candidatus Glomeribacter gigasporarum* (CaGg) and Mre, a coccoid Mollicutes-related endobacteria [2], while *Alternaria mali*, was reported to host *A. junii* and uncultured bacteria [1].

Endobacteria as symbionts may influence all phenotypic traits of their fungal host such as morphology, reproduction, growth, survival, resistance to stress, and behaviour including fungal pathogenicity [3]. CaGg have an important role on the pre-symbiotic phase of *Gigaspora* to its host.
CaGg removal from the host reduced proliferation of host hyphae [2]. Endobacteria may alter the host’s metabolites so that the morphology and behaviour of the host change [3]. Burkholderia rhizoxinica and B. endogungorum, endobacteria in Rhizopus microsporus determines the behaviour of R. microsporus [4] and produces rhizoxin (mycotoxin) as virulence factor [5]. Candidatus Glomeribacter gigasporarum and Rhizobium radiobacter (syn. of Agrobacterium tumefaciens) affects the morphology of Piriformospora indica and its ability to interact with its host plants as mycorrhiza [6-7]. Ralstonia solanacearum, a causal agent of wilt in various plants can become an endosymbiont of Fusarium oxysporum [8].

Fusarium oxysporum f.sp. cubense (Foc), a causal agents of Panama disease in bananas was reported carrying endobacteria [9]. Foc becomes one of the greatest threats to banana production in the world [10], including in Indonesia [11]. Foc infects host plants through roots and penetrates xylem vessels [12]. The pathogenicity of Foc is also influenced by the toxin. It produces fusaric acid (FA) [13]. FA plays an important role in triggering senescence in plants. The FA distributed in leaf, pseudostem, and root but mostly accumulated in leaves, exactly lower leaves [12]. FA changes the cell membrane potential balance [14]. There were 17 Foc strains from banana trees showing fusarium wilt symptom in Sumatra and Java that were reported to carry endobacteria in their conidia and hyphae [9]. However, the identity and role of endobacterial symbiont of Foc are unknown. The identity and role of endobacteria in Foc would help to describe the interaction between Foc and its host. This study aims to identify Foc endobacterial symbiont and to study the effect of symbiosis on the pathogenicity of Foc towards banana cv. Ambon Kuning banana and Tanduk.

2. Methods

2.1. Fungal cultures
Single-spore cultures of seven strains of Fusarium oxysporum f.sp. cubense (IPBCC 19.1427, IPBCC 19.1430, IPBCC 19.1434, IPBCC 19.1438, IPBCC 19.1439, IPBCC 19.1452 and IPBCC 19.1454) of IPBCC (IPB Culture Collection) collections were used in this study. The Exobacteria was firstly removed from the fungus using Van Tieghem method [15]. Exobacterial free hyphae were transferred on potato dextrose agar (PDA) and two days old culture was used for endobacterial detection and pathogenicity tests.

2.2. Detection of Foc endobacteria
The endobacteria were detected using a molecular approach and confirmed by staining method. The DNA was extracted using the protocol [16] with a modification in the lysis buffer solution. Sodium dodecyl sulfate was used as a lysis buffer. Mycelia were transferred into a 1.5 mL Eppendorf tube and were crushed using a pestle. About 500 µL sodium dodecyl sulfate (SDS) was added into the tube and incubated for 30 minutes at 65°C. Further, 500 µL of chloroform isoamyl alcohol (CI) was added and then the mixture was centrifuged at 12000 rpm for 15 minutes. About 300 µL of the supernatant was transferred into a new tube and 500 µL phenol chloroform isoamyl alcohol (PCI) was added. The mixture was centrifuged at 12000 rpm for 5 minutes in 4°C. About 300 µL of supernatant was then pipetted to the new tube and 500 µL of ethanol 99.5% and 50 µL of 2 M NaOAc were added into the tube. The mixture was incubated at -20°C. The mixture was centrifuged at 15000 rpm for 30 minutes. Ethanol 70% was added to the pellet and the mixture was then centrifuged at 10000 rpm for 5 minutes. The supernatant was removed and the pellet was dried. About 50 µL Nuclease Free Water (NFW) and 10 µL of RNAse 1 mg/mL were added to the dried pellet. The mixture was incubated at 37°C for 10 minutes and deactivated at 70°C for 10 minutes. DNA purity was measured using a Nanodrop Spectrophotometer. The extracted DNA was stored at -20°C until use.

The universal primers for bacteria, 27F/1492R primer [17] was used in this study for amplification of the 16S rDNA region. Amplification was performed on 10 µL of the total mix reaction consisting of 5 µL MyTaq HS Red Mix 2x, 0.5 µL primer, 27F 10 pmol, 0.5 µL primer 1492R 10 pmol, 1 µL DNA template, and NFW was added to reach volume 10 µL. Amplification conditions based on the protocol
[18] with modification at annealing temperature, i.e. initial denaturation at 96° for 5 minutes, followed by 33 cycles of denaturation at 96° for 1 minute, annealing at 53° for Foc IPBCC 19 1427 and 49.8° for Foc IPBCC 19.1430 for 1 minute, elongation at 72° for 2 minutes. The PCR results were electrophoresed at 100 Volt for 25 minutes using 1% agarose gel which was added with the 1st Fluorosafe Base in 1x TAE buffer. PCR products are visualized with Imaging SYNGENE Gel.

The presence of the endobacteria was done by transferring Foc hyphae onto a new slide glass. Hyphae were dripped with 100 µL LIVE/DEAD® BacLight™ Bacterial Viability Staining Kit [19] and incubated in darkness for 30 minutes. The hyphae were observed using fluorescence microscopes (BX51, Olympus, Tokyo, Japan) equipped with DLSR cameras (E-620, Olympus) at wavelengths of 460-490 nm. Green or red fluorescence in the fungal hyphae indicates the presence of the endobacteria. Living bacteria showed green fluorescence, while dead bacteria showed red fluorescence.

2.3. Identification of the endobacteria
Foc strains that positively contained endobacteria through staining and molecularly were further processed for sequencing. Only endobacteria with the clear band were going to be identified. The PCR product was sent to 1stBase Malaysia for sequencing. Endobacterial sequences were edited with ChromasPro version 1.7.7 and aligned with sequential data in GenBank through the Basic Local Alignment Search Tool (BLAST). Sequences were processed using MEGA 7 software. The phylogenetic tree showed the kinship between sequences. The name of endobacterium was determined based on the kinship of endobacterial sequences with sequence data in the database supported by bootstrap values> 95%.

2.4. In vitro pathogenicity testing of Foc towards banana plantling
The effect of endobacteria towards their fungal host was determined by comparing the pathogenicity of Foc- (containing endobacteria) and Foc+ (presumably free from endobacteria) towards plantlings of two banana cultivars, namely Ambon Kuning and Tanduk. Plantlings that were ready for acclimatization were used in this experiment. The plantlings were inoculated with either filtrate or biomass suspension of 21 days old each culture. Foc- cultures were obtained by growing 3 mycelial plugs in 100 mL PDB without antibiotics (ampicillin 500 mg/L and chloramphenicol 500 mg/L), while Foc+ were those of growing in antibiotics supplemented PDB. Both cultures were incubated in a static condition. The presence of endobacteria in the mycelial culture was observed by Gram staining. A small amount of biomass was crushed in a 1.5 mL Eppendorf tube and was then centrifuged 1000 rpm for 2 minutes. The supernatant was transferred to the new tube and re-centrifuged at 10000 rpm for 20 minutes. The supernatant was removed and the pellet was mixed with 500 µL NFW. A drop of pellet suspension was placed onto the surface of new slide glass and stained following Gram staining protocol [20]. The slides were observed under microscope at 1000 times magnification.

At the end of the incubation period, the filtrate was separated from biomass using Whatman® millipore (0.2 µm pore size). About 2 mL culture filtrate and culture filtrate prior to sieving (hereinafter referred to as biomass suspension) were transferred into the growing media of banana plantlings. PDB, the antibiotics supplemented PDB, and un-inoculated plantlings (controls) were used for comparison. Each treatment was done in 9 replicates (nine bottles, each containing one plantling). Symptoms that appear on the leaves and rhizomes were observed 10 days after inoculation (dai).

Pathogenicity was expressed in percentage of disease severity (DS) obtained through the conversion of scores of Leaf Symptoms Index (LSI) and Rhizome Discolouration Index (RDI) [21]. The LSI scale consists of 5 categories, i.e. score= 1 (the leaves are not yellow, look healthy), 2 (the 2 bottom leaves are slightly yellow or there are yellowing lines), 3 (there are at least 3 yellowing leaves), 4 (more than 3 yellow leaves or all leaves turned yellow), and 5 (dead plants). The RDI scale consists of 8 categories, i.e. score= 1 (no discolouration in rhizome and stele), 2 (no discolouration in rhizome, but rhizome and root discoloured), 3 (≥5% stele area changes colour), 4 (6-20% of the stele
area changes colour, 5 (21-50% of the stele area changes colour), 6 (> 50% of the stele area changes colour), 7 (discolouration in all parts of rhizome and stele), and 8 (dead plants).

2.5. Data analysis
Data were analysed based on multivariate analysis. The difference between the treatments was further tested by Tukey Honest Significant Difference at $\alpha = 0.05$ in SPSS 16.0 software. DS was calculated using the formula:

$$DS = \frac{\sum (n_i \times V_i)}{(N \times Z)} \times 100\%$$

Description:
- DS: disease severity
- Vi: scale LSI or RDI from plantling
- ni: number of plantling on a certain scale in one group
- Z: the highest scale value of LSI or RDI
- N: the total number of plants were tested

3. Result and discussion

3.1. The Endobacteria of Foc
All Foc strains hosted endobacteria (figure 1). The size of the endobacterial amplicon was in the range of 1400-1500 bp and was close to 16S rDNA Escherichia coli size of 1450 bp [22]. Endobacterial amplicons of Foc IPBCC 19.1427 and Foc IPBCC 19.1430 were sequenced. The presence of these endobacteria was confirmed with the fluorescence staining.

![Figure 1. Electrophoreogram of endobacterial 16S rDNA region of Foc. M1=marker 1 kb, M2= marker 100 bp, 1-8: Foc IPBCC 19.1427, Foc IPBCC 19.1430, Foc IPBCC 19.1434, Foc IPBCC 19.1438, Foc IPBCC 19.1439, Foc IPBCC 19.1452, Foc IPBCC 19.1454 and Escherichia coli (positive control).](image)

All Foc strains showed bacterial fluorescence in the hyphae, for example in Foc IPBCC 19.1438 (figure 2). Living bacteria showed green fluorescence, while the red fluorescence showed by dead bacteria [23]. However, mitochondria and fungal nucleus may also exhibit the green and red fluorescence, respectively. Thus, detection using LIVE / DEAD® BacLight™ Bacterial Viability Kit needs to be accompanied by molecular detection [1].

The phylogenetic analyses of endobacterial symbiont of Foc indicated that Foc IPBCC 19.1427 and Foc IPBCC 19.1430 hosted two different species of endobacteria and both endobacteria belongs to Enterobacteriaceae (figure 3). The endobacteria of Foc IPBCC 19.1427 was closely related to Enterobacter sp. since it was placed in one clade with Enterobacter spp. During BLAST search, the sequence of the endobacteria of Foc IPBCC 19.1427 had 92.98% similarity with Enterobacter sp. S11 [24].

Phylogenetic analysis placed Foc IPBCC 19.1430 endobacteria in one clade with Izhakiella australiensis (figure 4) that has strong support (BS=100%). Pantoea spp. (Enterobacteriaceae) which were found as endobacteria in Dothideomycetes and Sordariomycetes [1] is a sister clade of Izhakiella.
In contrast, another endobacteria in *Pestalotiopsis besseyi* (Sordariomycetes) i.e. *Bacillus atrophaeus* [1] is distantly related to *I. australiensis*

**Figure 2.** Hyphae of *Foc* IPBCC 19.1438 that had been stained by LIVE/DEAD® BacLight™ Bacterial Viability Staining Kit that was observed with (left) and without fluorescence filter (right) under fluorescence microscope. Scale bar: 20 µm.

**Figure 3.** Phylogenetic tree of 16S rDNA endobacteria of *Foc* IPBCC 19.1427 and *Foc* IPBCC 19.1430. Tree was reconstructed using Neighbour Joining (NJ) method with p-distance model. Bootstrap (BS)>50 was showed on the branch.

The Phylogenetic tree was reconstructed using to 16S rDNA of known fungal endobacteria. *Enterobacter* is Gram-negative and rod-shaped bacteria that are classified as facultative anaerobes [25]. Endohyphal *Enterobacter* is present in *Rhizoctonia solani* [26]. *Izhakielia australiensis* that was also Gram negative and rod-shaped bacterium was firstly described on the bases of isolates from sandy soil near Glendambo, Australia [25]. Prior to *I. australiensis* finding, the genus consisted of one
species, namely *I. capsodis* from *Capsodes infucatus* (Hemiptera: Miridae). The Mirid bug contained 3 bacterial strains similar (94.7-95.7%) to *Pantoea* (Enterobacteriaceae), but the phenotype and phylogenetic analysis were closer to *Izhakiella* [27]. *Izhakiella australiensis* as endobacteria has never been reported prior to this study, so this study is the first report of endohyphal *I. australiensis* in *Foc*. *Enterobacter* and *Izhakiella* (Enterobacteriaceae) are in different family with other known endohyphal bacteria such as *Acinetobacter* (Moraxellaceae) and *Burkholderia* (Burkholderiaceae). Other endohyphal bacteria such as *Bacillus* and *Paenibacillus* (Bacillales) were distantly related and were used as outgroup.

3.2. The role of endobacteria on the pathogenicity of *Foc*

Two (*Foc* IPBCC 19.1427 and *Foc* IPBCC 19.1430) among the 7 *Foc* strains carrying endobacteria were selected to be tested for their pathogenicity. The role of the endobacteria on the pathogenicity of *Foc* can be studied by comparing the pathogenicity of the same strain of *Foc* that contained endobacteria from those free from endobacteria. Therefore, culturing *Foc* carrying endobacteria in antibiotics supplemented media was expected to suppress bacterial populations in hyphae to zero or a level that they occurrence did not affect the host. The occurrence of the endobacteria was thus analyzed. The results showed that *Foc* IPBCC 19.1427 biomass still contained Gram-positive and negative bacteria (Figure 4 left), while the *Foc* 19.1430 pellet suspension contained Gram-negative bacteria (Figure 4 right). The presence of Gram-positive bacteria in *Foc* IPBCC 19.1427 was not detected during the molecular identification. In contrast, the presence of Gram-negative endobacteria in *Foc* IPBCC 19.1427 and *Foc* IPBCC 19.1430 biomass was supported by the results of molecular identification, namely *Enterobacter* sp. and *I. australiensis*, respectively.

The addition of antibiotics apparently suppressed the bacterial population but did not completely kill the bacteria. The antibiotic used in this study was five times of ampicillin concentration for axenic fungus culture containing endohyphal bacteria [28]. Instead of using 500 ppm for ampicillin and chloramphenicol, they used 50 ppm kanamycin, 10 ppm tetracycline, and 40 ppm ciprofloxacin, in addition to ampicillin 100 ppm.

![Endobacterial Gram staining of *Foc* IPBCC 19.1427 (left) and *Foc* IPBCC 19.1430 (right). Scale bar: 10 µm.](image)

Filtrate and biomass suspension given to plantling caused leaf senescence (figure 5 above). Leaf senescence was caused by *Foc* infection [10]. The symptom appeared starting from 4 dai. These treatments also caused rhizomes discoloration [29] and observed at 10 dai (figure 5 below). The disease severity due to filtrate treatment was generally lower and significantly different from those of
biomass suspension. The mycelium of *Foc* in biomass suspension began to grow at 2 dai and continuously grow to cover the surface of the media at the end of the incubation period. This condition allowed the living biomass to produce effectors continuously while the effectors in the filtrate given were available only in a certain amount. The filtrate was suspected to contain toxins. The presence of toxins such as fusaric acid (FA) accelerated the appearance of leaf senescence [10]. FA might interact with other secondary metabolites such as moniliformin and fumonisin [11] and interaction of mycotoxin with FA increased toxicity [30]. In the host plant, FA was secreted after *Foc* mycelia reached the xylem vessel and carried along with the water to the leaf. FA changed membrane balance, inhibited ATP formation, decreased mitochondrial activity, and oxygen uptake [12]. FA activity accelerates the senescence of banana plant leaves [10].

![Figure 5](image)

Figure 5. Leaf senescence symptom (above) and rhizome discolouration (below) that was caused by *Foc* filtrate (left), *Foc* biomass treatment (middle), comparing to untreated plantling (control) at 10 day after inoculation.

Disease severity in cv. Ambon Kuning (figure 6) and cv. Tanduk (figure 7) due to *Foc* IPBCC 19.1427 treatment varied. In cv. Ambon Kuning, DS due to *Foc* IPBCC 19.1427- biomass suspension treatment was greater and significantly different from that of *Foc* IPBCC 19.1427 + biomass suspension treatment, but the administration of its filtrate- was not significantly different from that of filtrate+. This indicates that DS was not caused by metabolites in the filtrate but presumably by other pathogenicity mechanism controlled by host-pathogen cells communication. In cv. Tanduk, DS caused by *Foc* IPBCC 19.1427 biomass- and filtrate- treatment were higher and significantly different that of biomass+ and filtrate+ treatment (figure 7). Thus, it is suspected that endobacteria played a role in effectors production that lead to symptom formation in cv. Tanduk. Endobacteria in *R. microspores, B. rhizoxinica* was found to have a role in the production of toxins from its host [3]. Previously, it was stated that toxin production is highly dependent on the bacterial strain of endosymbionts [16].

In contrast to the *Foc* IPBCC 19.1427, *Foc* 19.1430 endobacteria did not affect disease severity in leaf senescence in cv. Ambon Kuning (figure 8) and Tanduk (figure 9) as disease severity caused by *Foc* 19.1430 biomass- and filtrate- treatment was not significantly different from those of *Foc* 19.1430 biomass+ and filtrate+ treatment. In cv. Tanduk, the degree of rhizome discoloration caused by *Foc* 19.1430 biomass- treatment was higher than that of *Foc* 19.1430 +, but the percentage of symptoms in leaves and filtrate treatment were not significantly different. Therefore, *Foc* 19.1430 symbiont endobacteria are thought not to affect the pathogenicity of *Foc* 19.1430 to both banana cultivars. Symbiotic endobacteria on Zygomycetes which had no effect on mucormycosis [16].

In general, DS due to the provision of PDB and PDB+ was higher, although it was not significantly different from the control. This is thought to be related to sugar content in PDB. The sugar level in the planting environment (PDB treatment) was higher than the control. In a balanced and healthy condition, the addition of sugar levels could change the osmotic balance of plant cells and got senescence. Deficiencies and excess sugar levels caused senescence [31].
Figure 6. Disease severity on cv. Ambon Kuning caused by *Foc* IPBCC 19.1427 treatment, based on leaf severity index (left) and root discoloration index (right). Standard error is shown by vertical line on each bar and the letters indicates mean difference obtained from Tukey HSD test at $\alpha=0.05$.

Figure 7. Disease severity (DS) in cv. Tanduk caused by *Foc* IPBCC 19.1427 treatment based on leaf severity index (left) and root discolouration index (right). Standard error is shown by vertical line on each bar and the letters indicates mean difference obtained from Tukey HSD test at $\alpha=0.05$. 
Figure 8. Disease severity on cv. Ambon Kuning caused by *Foc* 19.1430 treatment based on leaf severity index (left) and root discolouration index (right). Standard error is shown by vertical line on each bar and the letters indicates mean difference obtained from Tukey HSD test at $\alpha=0.05$.

Figure 9. Disease severity on cv. Tanduk caused by *Foc* 19.1430 treatment based on leaf severity index (left) and root discolouration index (right). Standard error is shown by vertical line on each bar and the letters indicates mean difference obtained from Tukey HSD test at $\alpha=0.05$.

4. Conclusion

Seven *Foc* strains being studied contained endobacteria. *Enterobacter* sp. was the endobacteria hosted by *Foc* IPBCC 19.1427 and *Izhakiella australiensis* was hosted by *Foc* 19.1430. *Foc* IPBCC 19.1427 endobacterial symbiont effected *Foc* pathogenicity towards cv. Tanduk through effector production in
the culture filtrate, and through other mechanisms on Ambon Kuning. *Foc* 19.1430 symbiotic endobacteria did not affect disease severity on both banana cultivars. The disease severity obtained is under the condition where the antibiotic amended to the production media could not completely kill the endobacteria.

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