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Molecular and Biochemical Detection of *Fusarium oxysporum* f.sp. *cubense* as the Pathogen of *Fusarium* Wilt Disease on Banana (*Musa* spp.)

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ABSTRACT

Molecular and biochemical characteristics of Fusarium oxysporum f.sp. cubense (Foc) were detected. Six of Indonesian Foc isolates were artificially inoculated on "Ambon Kuning" banana. DNA of one-week culture isolates was extracted by three methods prior to PCR assay using Foc TR4 (tropical race 4) specific primer. Activity of extracellular enzyme was determined with reduction sugar, agar diffusion and SDS-PAGE assays. Statistical analysis revealed that all isolates insignificantly caused Fusarium wilt symptoms on tested banana with disease severity index ranging from 3 to 3.6. Maximum DNA concentration was obtained by CTAB method (766.25 µg mL^{-1}), followed by SDS and alkaline lysis methods, i.e. 553.75 and 211.25 µg mL^{-1}, respectively. PCR analysis showed that Bnt2 and Kjg1 isolates positively reacted to TR4 of Foc primer (DNA size of 1400 bp approximately). Reduction sugar and agar diffusion assays demonstrated that Kjg1 isolate significantly produced more extracellular enzyme, with 6.53 × 10^{-2} mg mL^{-1} in concentration and 20 mm in halo diameter. Meanwhile, SDS-PAGE assay viewed diverse bands of tested fungi (20.6 to 80 kDa), representing four extracellular enzymes. Positive PCR results highlighted the presence of Foc TR4 infecting banana in Indonesia. Various activities of extracellular enzymes did not influence the pathogenicity of Foc.

Key words: pathogenicity, DNA concentration, extracellular enzyme

INTRODUCTION

As a popular fruit in tropical countries, banana production in Indonesia reached about 2.5 million tons a year (Wibowo *et al.*, 2004). The development of large-scale cultivation in some areas such as Halmahera Island (North Maluku), Lampung, Mojokerto (East Java), could increase the export volume of Indonesian banana to be 100 000 tons in 1996, but then drastically decrease to 27 tons in 2004 (Badan Penelitian dan Pengembangan Pertanian, 2005).

One of the most significant threats to banana production worldwide is *Fusarium oxysporum* f.sp. *cubense* (*Foc*) causing *Fusarium* wilt (Panama disease) (Bentley *et al.*, 2001). In Indonesia, this pathogen had been a problem in 25 of provinces including 115 regencies with high severity and the disease had widely spread into thousands of banana plantations for three consecutive years (2001- 2003), namely over 1 491 580 to 2 781 029 ha (Widodo, 2004).

Based on its pathogenicity level to banana cultivars, this fungus is classified into four physiological races, i.e. race 1 attacking 'Gros Michel' (AAA) and 'Lady finger' (AAB) cultivars; race 2 infecting 'Bluggoe' (ABB) cultivar; and race 4 harassing Cavendish (AAA) cultivar and those cultivars which were susceptible to races 1 and 2 (Ploetz, 1993). In addition to causing enormous economic losses, this pathogen is quite difficult to control because it has chlamydospores as its main inoculum which can survive for a long time in plant debris and soil (CABI, 2003). Nasir and Jumjumidang (2004) noticed that it was important to understand its characteristics in order to develop proper control strategies. This study, therefore, was carried out to detect physiological characteristics of *Foc* isolates in Indonesia through molecular analysis and activity of their extracellular enzymes.

MATERIALS AND METHOD

Six isolates of *Foc* collected from infected bananas on some locations in Indonesia (Table 1) were artificially inoculated on two months seedlings of "Ambon Kuning" cultivar of banana plants (AAA) with 10^6 conidia mL^{-1} to assess their pathogenicity levels under glass house condition. Disease severity index (DSI) was measured by observing and scoring the symptoms which

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appeared on the vertical section of pseudo-stem of tested plants with this following equation at sixth week after inoculation (Ho *et al.*, 2004):

$$DSI = \frac{\sum(nxv)}{\sum(N)}$$

(nxv) = symptomatic plants with their corresponding score
N = total number of tested plants

For molecular detection, the DNA from one-week fungal cultures in potato dextrose broth (PDB) medium were extracted by three different extraction methods, namely Alkaline (Lee *et al.*, 2006), CTAB (Subandiyah, 2003), SDS (KSU Research and Extension, 2006) methods with slight modification. Afterwards, PCR analysis was run using a pair of specific primer for tropical race 4 (TR4) of *Foc* according the combination and modification of methods developed by Bentley *et al.* (2001) and Cooperative Research Centre for Tropical Plant Protection (2003).

Table 1. *Foc* isolates used in this study

Isolates	Race	Geographic Origin	Host and genotype	Collector
Bnt1	4	Central Java	Cavendish (AAA)	Arif Wibowo
Bnt2	4	Central Java	Pisang Awak (ABB)	Arif Wibowo
Btu3	4	East Java	Pisang Raja (AAB)	Arif Wibowo
Kjg1	4	East Kalimantan	Pisang Ambon (AAA)	Siti Subandiyah
Lmp3	4	Lampung, Sumatra	Cavendish (AAA)	Arif Wibowo
Wsb3	4	Central Java	Pisang Ambon (AAA)	Arif Wibowo

To determine the activity of extracellular enzyme, four-day fungal cultures in PDB were harvested and their conidia concentrations were then adjusted to 10^6 conidia mL^{-1}. Those conidia suspensions were used for preparation of fungal filtrate following the method developed by Di Petro *et al.* (1998). Thereafter, the activity of extracellular enzyme was consecutively detected with reduction sugar (Sudarmadji *et al.*, 1997), agar diffusion (Raymond and Gary, 1988) and SDS-PAGE (Takacs and Kereses, 1984) assays.

These experiments were conducted under completely randomized design (CRD) with three replications. Data were statistically examined with analysis of variance (ANOVA) procedure using SAS® Systems for Windows V8 software (SAS Institute, Cary, North California; USA) and the means were compared with Duncan multiple range test (DMRT).

RESULTS AND DISCUSSION

The scoring of pathogenicity test was determined by observing the internal symptom on vertical section of pseudo-stems of inoculated banana. The severe lesion was produced by plants which were inoculated with Btu3 isolate, namely scoring 3.6. Based on rhizome discoloration index (RDI), Endah and Novizan (2002) illustrated that score as the necrotic lesion with more than 5% infection around pseudo-stem. They also described this index as the development of browning ring-like symptoms on pseudo-stem which could alternate into soft rot symptom.

Statistical analysis, however, revealed the insignificant DSI among those isolates ranging from 3, which were exposed by Bnt1 and Lmp3 isolates, to 3.6 by Btu3 isolate (Table 2). These results indicated that all tested *Foc* isolates had similar virulence level in causing *Fusarium* wilt disease on banana cultivar "Ambon Kuning" (AAA).

Extraction results showed that CTAB method could produce more DNA concentration (766.25 µg mL^{-1}) compared to SDS and Alkaline Lyses, namely 553.75 and 211.25 µg mL^{-1} in average, respectively (Table 3). According to Subandiyah (2003), the reagent components of former method could minimize protein contamination by precipitating protein, phenolic compounds, and polysaccharides which contaminated the expected DNA.

Only Bnt2 and Kjg1 isolates viewed positive reactions to a pair of specific primer for TR4 of *Foc*, with approximately 1400 bp of DNA in size (Fig. 1). These results documented the presence of tropical races 4 in Indonesia at a molecular level. Hennessy *et al.* (2004) described these races as the particularly virulent strain of *Foc* affecting Cavendish in the absence of cold stress in tropical areas of Indonesia and Malaysia and differed with

race 4 strain infecting Cavendish in the subtropics (which known as subtropical race 4). They also reported that TR4 of *Foc* has been detected in a commercial banana plantation in Darwin, North Territory (NT) of Australia on 1997.

Table 2. Disease severity index (DSI) of *Foc* isolates under pathogenicity test, the concentration of their enzyme activities detected using sugar reduction method and their halo diameter under diffusion agar assay

Isolate	DSI	Absorbance 540 nm	Concentration (mg mL^{-1})	Halo diamater (mm)
Bnt1	3a	0.0716	0.0273bc	14c
Bnt2	3.4a	0.1476	0.0556ab	16b
Btu3	3.6a	0.0883	0.0333abc	14.25c
Kjg1	3.4a	0.1713	0.0653a	20a
Wsb3	3.2a	0.1436	0.054ab	9.253
Lmp3	3a	0.0096	0.0036c	13d

Note: The number followed by same letter in one column indicated the insignificant different under DMRT at $\alpha = 5\%$

Table 3. Quantification data of DNA extracted from six isolates with three extraction methods at optical density (OD) 260 and 280 nm

Method	Isolates	OD_{260}	OD_{280}	Ratio of OD_{260} : OD_{280}	DNA concentration (µg mL^{-1})
CTAB	Bnt1	0.356	0.196	1.816	890
	Bnt2	0.534	0.318	1.679	1335
	Btu3	0.257	0.161	1.596	642.5
	Kjg1	0.215	0.158	1.361	537.5
	Lmp3	0.294	0.247	1.190	735
	Wsb3	0.183	0.102	1.794	457.5
Average		0.307	0.197	1.573	766.250
Alkaline Lysis	Bnt1	0.083	0.082	1.012	207.5
	Bnt2	0.012	0.017	0.706	30
	Btu3	0.058	0.052	1.115	145
	Kjg1	0.128	0.116	1.103	320
	Lmp3	0.222	0.42	0.529	555
	Wsb3	0.004	0.005	0.800	10
Average		0.085	0.115	0.878	211.250
SDS	Bnt1	0.25	0.159	1.572	625
	Bnt2	0.086	0.072	1.194	215
	Btu3	0.247	0.184	1.342	617.5
	Kjg1	0.355	0.225	1.578	887.5
	Lmp3	0.165	0.109	1.514	412.5
	Wsb3	0.226	0.17	1.329	565
Average		0.222	0.153	1.422	553.750

According to Handayani (2005), Bnt1 and Bnt2 isolates belonged to race 4 because these isolates could infect Ambon, Kepok, Cavendish, and *Heliconia* spp. under artificial inoculation in glasshouse condition. As they produced volatile compounds, all tested isolates in this study were physiologically categorized into odoratum types (Nasir and Jumjunidang, 2004). Previously, Wibowo *et. al.* (2002) reported that Bnt1 and Bnt2 isolates were odoratum types based on volatile odour test; whereas according to vegetative compatibility group (VCG) test, Bnt1 was grouped into 0120 or 0129.

Figure 1. PCR results of *Foc* isolates with specific primer TR4 of *Foc*. Lane (M) DNA marker; (1) Bnt1, (2) Bnt2, (3) Wsb3, (4) Btu3, (5) Kjg1 and (6) Lmp3 isolates

The maximum activity of extracellular enzyme was performed by Kjg1 isolate, showing utmost concentration under reduction sugar (6.53×10^{-2} mg mL^{-1}) and maximum halo diameter under agar diffusion assays (20 mm) (Table 2). Meanwhile, various activities were visualized by SDS-PAGE with range of 20.6-80 kDa which corresponded to four different extracellular enzymes, namely lysozyme (20.6 kDa) produced by Btu3 isolate, carbonic anhidrase (34.8 kDa) by Bnt2, ovalbumin (49.1 kDa) by Lmp3 and bovin serum albumin (80 kDa) (Fig. 2).

Those results, however, did not reveal any extracellular protein correlating with pathogenicity levels. Di Petro *et al.* (1998) reported that polygalacturonase, extracellular enzyme which contributed to virulence level of fungal pathogen, could be subsequently observed on 35 and 37.5 kDa under SDS-PAGE assay.

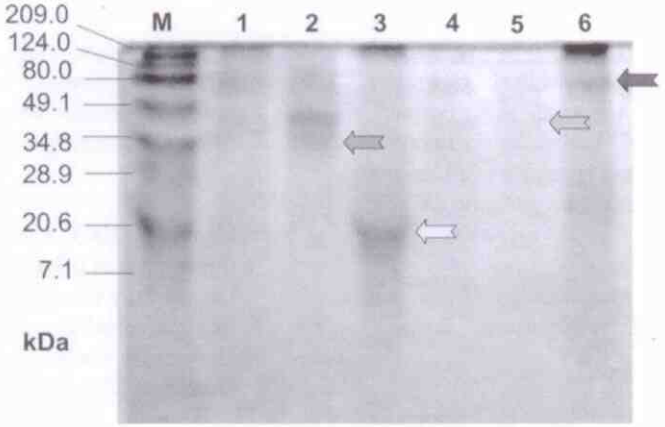

Figure 2. Bands of extracellular protein from *Foc* isolates viewed with SDS-PAGE method. Lane (M) marker, (1) Bnt1, (2) Bnt2, (3) Btu3, (4) Wsb3, (5) Lmp3, and (6) Kjg1 isolates.

CONCLUSION

The presence of tropical race 4 of *Foc* in infecting banana plantation in Indonesia was notified by positive responses of Bnt2 and Kjg1 isolates upon the specific primer for TR4 of *Foc* under PCR assay. The comparable virulence levels of *Foc* isolates were not affected by various activities of their extracellular enzymes.

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