Occurrence of Tropical Race 4 of *Fusarium oxysporum* f. sp. *cubense* in Indonesia

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Received on January 25, 2011; Revised on June 13, 2011; Accepted on June 13, 2011)

Fusarium wilt of banana caused by *Fusarium oxysporum* f. sp. *cubense* is widespread in Indonesia. However, the distribution of tropical race 4 strains has not been well studied. Thirty nine isolates of *F. oxysporum* f. sp. *cubense* were collected from Java and 7 isolates were from Sumatera, Bangka, and Kalimantan. All isolates produced volatile odor when grown on steamed rice. These isolates were further tested for their vegetative compatibility with *nitM* testers of 20 reported vegetative compatibility groups representing strains that belong to race 1, 2, and 4. Three isolates formed heterokaryons with *nitM* testers belong to race 1, 11 isolates with race 4, and the rest did not form heterokaryons with all *nitM* testers used. *F. oxysporum* f. sp. *cubense* tropical race 4 specific primer pair was used to amplify a 1400 bp fragment of tropical race 4 DNA. Seven isolates (Bnt2, Mln1, Srg1, Bgl3, Bgl6, Lmp1, and Kjj1) produced the 1400 bp amplification product were therefore tropical race 4.

Keywords : *F. oxysporum* f. sp. *cubense*, tropical race 4, vegetative compatibility groups

Fusarium wilt of banana is a serious problem for banana production in many areas of the world (Stover, 1993). In Asia, fusarium wilt is widespread in many countries such as India, Indonesia, Malaysia, Taiwan, and the Philippines (Bentley et al., 1998). Fusarium wilt of banana was first reported in Indonesia in 1916 from banana plantations in Java (Stover, 1962a) and has since been found to be widespread in many banana production areas of Indonesia. Based on a survey conducted by Indonesian Centre of Agricultural Quarantine, fusarium wilt of banana has been found in Sumatera, Java, Bali, Nusa Tenggara, Kalimantan, Sulawesi, Maluku, and Papua (Semangun, 2004).

Fusarium wilt of banana is caused by *Fusarium oxysporum* Schlecht. f. sp. *cubense* (E.F. Smith) Snyder and Hansen (Ploetz and Pegg, 2000). Four races of *F. oxysporum*

f. sp. *cubense* are currently recognized based on their pathogenicity to different banana cultivars. Race 1 of *F. oxysporum* f. sp. *cubense* is virulent to Gros Michel (AAA); race 2 is virulent to Bluggoe (ABB); race 3 is virulent to Heliconia and race 4 is virulent to Cavendish and all cultivars susceptible to races 1 and 2. The Cavendish cultivars were affected mainly in the subtropics. Recently, fusarium wilt has also devastated Cavendish cultivars grown for export in tropical regions. The isolate of *F. oxysporum* f. sp. *cubense* primarily responsible for this devastation is called tropical race 4 (Ploetz, 2005).

Vegetative compatibility is the ability of any two strains to form heterokaryons with each other (Puhalla, 1984). Ploetz and Corell (1988) showed that, in general, there is a good correlation between races and vegetative compatibility groups (VCGs) among isolates for which the race is known. Worldwide, there are at least 21 VCGs of *F. oxysporum* f. sp. *cubense* with the number of VCGs being greatest in Asia where it is believed that *F. oxysporum* f. sp. *cubense* evolved (Bentley et al., 1998). Other criteria have been used to identify variation within *F. oxysporum* f. sp. *cubense*. Stover (1962b) cultured *F. oxysporum* f. sp. *cubense* isolates on steamed rice and classified as odoratum or inodoratum isolates based on their production of a strong volatile odor. Bentley et al. (2003) has developed a DNA diagnostic test specific for tropical race 4 of *F. oxysporum* f. sp. *cubense* based on DNA sequence information that is unique to the tropical race 4 of *F. oxysporum* f. sp. *cubense*. Database searches of DNA sequence information published in GenBank have indicated that there are no matches for these primers with any other organisms, and extensive laboratory screening confirms the specificity of the tropical race 4 primers.

It has previously been considered that only *F. oxysporum* f. sp. *cubense* race 1 was present in Indonesia, affecting the local banana cultivars such as Pisang ambon, barangan, raja, susu and mas. However, Nasir et al. (2003) found that fusarium wilt of banana has also destroyed Cavendish cultivar which is resistant to race 1 of *F. oxysporum* f. sp. *cubense* in areas such as Riau (300 ha), Halmahera (3000 ha), and Lampung (1700 ha). This indicates that other races

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of *F. oxysporum* f. sp. *cubense* may occur in Indonesia. This study aimed to identify tropical race 4 isolates of *F. oxysporum* f. sp. *cubense* in Indonesia by assessing volatile production, vegetative compatibility groups (VCGs) and a tropical race 4 PCR-based detection test.

Isolate collection. Isolates of *F. oxysporum* f. sp. *cubense* were collected from Java, Sumatera, Bangka, and Kalimantan. The cultivars of diseased banana plants and their location are presented in Table 1. Isolation of *F. oxysporum* f. sp. *cubense* from banana plants was made by cutting out a portion of the necrotic vascular tissue of the pseudostem. The tissue pieces were put onto acidified PDA medium and incubated at room temperature for 7 days. Colonies growing on the medium were observed microscopically, and those identified as *F. oxysporum* were transferred to fresh PDA medium. Single spore isolation was made from each colony, and the identity of each isolate was confirmed morphologically based on the morphology of the conidia produced (Leslie and Summerell, 2006). The pure cultures of *F. oxysporum* were preserved on PDA slants in sterile liquid paraffin, and stored at 28°C. A total of 46 isolates of *F. oxysporum* were collected from several different banana varieties. Thirty nine isolates of *F. oxysporum* were collected from Java and a further 7 isolates were from Sumatera, Bangka, and Kalimantan (Table 1). Pathogenicity test was conducted on susceptible banana plantlets, i.e., Pisang raja sereh (AAB) cultivar in order to determine *F. oxysporum* f. sp. *cubense*. Three banana plantlets grown on MS medium were inoculated with 7 days old mycelial disc of fungal isolates by placing the disc onto the medium and incubated for 4 weeks at 25°C. Observation of rotting plantlets was conducted every week. The result showed that banana plantlets inoculated with the 46 fungal isolates became necrotic mostly after one week of incubation and completely rotten after 3 weeks. This indicated that all fungal isolates collected were pathogenic *F. oxysporum*.

Production of volatile metabolites on rice medium. All isolates of *F. oxysporum* f. sp. *cubense* were cultured on steamed rice (25 mL of rice and 50 mL of water added into 100 mL Erlenmeyer flasks, which were plugged with cotton-wool and sterilized by autoclaving at 121°C for 20 minutes). Cultures were grown at 28°C. After 10 days, cultures were classified based upon the presence of a strong volatile odor (Moore et al., 1991). From the 46 isolates of *F. oxysporum* f. sp. *cubense* tested, 20 isolates (Bjr2, Bdg1a, Kph, Kp1, Gn3, Mln1, Bgl3, Bgl6, Wsb5, Srg1, Kdg2, Btu3, Pjn4, Pjn5, Lmp1, Lmp3, Bnk12, Bnk25, Kjg2, Pkr1) produced a strong benzolic odor and 26 isolates (Bnt1, Grt1, Grt3, Kp4, Gn2, Bnt1, Bnt2, Kd1, A13, Tmg1,

Wsb1, Wsb3, Mgl1, Mgl2, Mgl6, Sdt1, Pbn2, Skj1, Skj2, Kdg1, Btu3a, Btu4, Kmr1, Kbr1, Pkl1, Kjg1) produced a “nutty” odor when cultured on steamed rice medium and classified as odoratum types (Table 1). Stover (1962) showed that isolates from Bluggoe, which were placed in race 2, did not produce an odour, whereas isolates in race 1 belonged to either odoratum or inodoratum. However, Moore et al. (1991) showed that the production of volatiles on steamed rice by Australian isolates of *F. oxysporum* f. sp. *cubense* correlated well with common vegetative compatibility groups of race 4 isolates. Thus, this study suggested that race 1 and/or race 4 of *F. oxysporum* f. sp. *cubense* was present in Indonesia.

Vegetative compatibility analysis. Single spore isolates of all odoratum isolates were grown on minimal agar medium with chlorate (MMC) containing 15 g/L of KClO₃ (Correll et al., 1987). The growth of most isolates was slow and limited to the surface of the medium. After 1–3 weeks, chlorate-resistant sectors (putative *nit* mutants) arose from these colonies. These *nit* mutants were recognized by their fluffy, aerial mycelium and a rapid growth rate comparable with the wild-type colonies on PDA. To confirm that the sectors were *nit* mutants, mycelium from the sectors was grown on Puhalla’s minimum medium (MM) (Ploetz and Correll, 1988). The *nit* mutants were identified by their thin, expansive growth on this medium. Each *nit* mutant of each odoratum isolate was paired with *nit*M VCG testers which consisted of the 20 reported VCGs (0120, 0121, 0122, 0123, 0124, 0125, 0126, 0128, 0129, 01210, 01211, 01212, 01213, 01214, 01215, 01216, 01217, 01218, 01219, and 01220) representing strains that belong to race 1, 2, and 4. Each odoratum isolate was tested against each tester on MM. The *nit* mutants that were able to anastomose with the testers formed a line of wild-type growth where the two colonies came into contact.

The result showed that 3 isolates (Bnt1, Mlg6, and Pkr1), each formed heterokaryon with 0123, 0124, and 0126, respectively. Thus, these 3 isolates belonged to race 1. Two isolates (Gn3, Wsb5) formed heterokaryons with 0120, one isolate (Pjn4) with 0129 and 7 isolates (Bnt2, Mln1, Srg1, Bgl3, Bgl6, Lmp1, and Kjg1) with 01213/16. All of these heterokaryons grew strongly. One isolate (A13) formed a weakly growing heterokaryon with 01213/16. Thus, these 11 isolates belonged to race 4. The rest did not form heterokaryons with all *nit*M VCG testers used and classified as VCG unknown (Table 1). Bentley et al. (1998) stated that isolates were classified as VCG unknown either because they had not yet been typed by VCG analysis prior to DNA finger printing or they were not compatible with the available set of *nit*M testers representing the 20 currently recognized VCGs.

Table 1. Distribution of banana cultivars showing fusarium wilt symptoms in Java, Sumatera, Bangka, Kalimantan islands, the production of volatile metabolites by isolates of *F. oxysporum* f. sp. *cubense* from these cultivars, and their vegetative compatibility groups (VCGs)

No.	Fungal isolates	Geographic origin	Host cultivar	Host Genotype	Volatile production ^a	VCG ^b
1	Btl1	West Java	Pisang raja sereh	AAB	+, N	nc
2	Bjr2	West Java	Pisang awak	ABB	+, B	nc
3	Grt1	West Java	Pisang ambon	AAA	+, N	nc
4	Grt3	West Java	Pisang ambon	AAA	+, N	nc
5	Bdg1a	West Java	Pisang ambon	AAA	+, B	nc
6	Kph	Central Java	Pisang kepok	ABB	+, B	nc
7	Kp1	Central Java	Pisang ambon	AAA	+, B	nc
8	Kp4	Central Java	Pisang ambon	AAA	+, N	nc
9	Gnk2	Central Java	Pisang ambon	AAA	+, N	nc
10	Gnk3	Central Java	Pisang ambon	AAA	+, B	0120
11	Bnt1	Central Java	Cavendish	AAA	+, N	0123
12	Bnt2	Central Java	Pisang awak	ABB	+, N	01213/16
13	Kd1	Central Java	Pisang kepok	ABB	+, N	nc
14	Mln1	Central Java	Pisang kepok kerau	ABB	+, B	01213/16
15	A13	Central Java	Pisang ambon	AAA	+, N	01213/16 ^{wh}
16	Bgl3	Central Java	Pisang kepok	ABB	+, B	01213/16
17	Bgl6	Central Java	Pisang raja	AAB	+, B	01213/16
18	Tmg1	Central Java	Pisang ambon	AAA	+, N	nc
19	Wsb1	Central Java	Pisang raja	AAB	+, N	nc
20	Wsb3	Central Java	Pisang ambon	AAA	+, N	nc
21	Wsb5	Central Java	Pisang ambon	AAA	+, B	0120
22	Mgl1	Central Java	Pisang raja	AAB	+, N	nc
23	Mgl2	Central Java	Pisang raja	AAB	+, N	nc
24	Mgl6	Central Java	Pisang awak	ABB	+, N	0124
25	Srg1	Central Java	Pisang raja	AAB	+, B	01213/16
26	Sdt1	Central Java	Pisang ambon	AAA	+, N	nc
27	Pbn2	Central Java	Pisang ambon	AAA	+, N	nc
28	Skj1	Central Java	Pisang ambon	AAA	+, N	nc
29	Skj2	Central Java	Pisang ambon	AAA	+, N	nc
30	Kdg1	East Java	Pisang kepok	ABB	+, N	nc
31	Kdg2	East Java	Pisang kepok	ABB	+, B	nc
32	Btu3	East Java	Pisang raja	AAB	+, B	nc
33	Btu3a	East Java	Pisang raja	AAB	+, N	nc
34	Btu4	East Java	Pisang kepok	ABB	+, N	nc
35	Pjn4	East Java	Pisang raja	AAB	+, B	0129
36	Pjn5	East Java	Pisang raja	AAB	+, B	nc
37	Kmr1	East Java	Pisang ambon	AAA	+, N	nc
38	Kbr1	East Java	Pisang ambon	AAA	+, N	nc
39	Pkl1	East Java	Pisang ambon	AAA	+, N	nc
40	Lmp1	Lampung Sumatra	Pisang raja nangka	AAB	+, B	01213/16
41	Lmp3	Lampung Sumatra	Cavendish	AAA	+, B	nc
42	Bnk12	Bangka	Pisang ambon	AAA	+, B	nc
43	Bnk25	Bangka	Pisang ambon	AAA	+, B	nc
44	Kjg1	East Kalimantan	Pisang ambon	AAA	+, N	01213/16
45	Kjg2	East Kalimantan	Pisang raja	AAB	+, B	nc
46	Pkr1	East Kalimantan	Pisang kepok	ABB	+, B	0126

^a+, N indicates a “nutty” odour, +, B indicates a strong “benzolic” odour; ^b nc indicates no compatibility occurred with any of the VCG testers used, ^{wh} indicates only a weak heterokaryon was formed

Diagnostic PCR. Eleven race 4 of Foc (Bnt2, Mln1, Srg1, Bgl6, Bgl3, Lmp1, Kjg1, A13, Pjn4, GnK3, and Wsb5) and other 3 odoratum (Btu3, Tmg1, and Bnt1) isolates were grown in 40 mL of potato dextrose broth (Burgess et al., 1994) in 100 mL Erlenmeyer flasks and shaken at 100 rpm at 28°C for 7 days. The contents of the flasks were filtered through filter paper to collect the mycelium which was then ground in buffer using a mortar and pestle. Total DNA was extracted using a buffer containing cetyltrimethylammoniumbromide (CTAB) according to protocol of Steen (1999). DNA was then dissolved in TE buffer and stored at -20°C. PCR was conducted as described by Bentley et al. (2003). The primer pair used in this study was TR4-F2 (5'-CGCCAGGACTGCCTCGTGA-3') and TR4-R1 (5'-CAGGCCAGAGTGAAGGGGAAT-3'). The final concentration of the primer pair in the PCR was 12.5 µM. DNA amplification was carried out using MegaMix-Royal (Microzone Limited) (Double concentrated PCR mix, containing optimized mixture of *Taq* polymerase, anti-*Taq* polymerase monoclonal antibodies in 2 x reaction buffer (6 mM MgCl₂) with 400 µM dNTPs, stabilizer and blue loading dye). The temperature cycling program used was as follows: an initial denaturation at 95°C for 2 min; followed by 30 cycles consisting of 95°C for 30 s, 68°C for 1 min 30 s and a final extension step consisting of 72°C for 3 min. The amplified products were subjected to electrophoresis at 100 V in a horizontal 1.5% agarose gel using 1xTBE buffer for 45 min. The amplification products were stained with ethidium bromide and visualized under UV light and photographed. A 100 bp DNA ladder (Promega) was used as a marker to aid in the determination of the sizes of the amplification products.

The PCR primer pair, TR4-F2 and TR4-R1, amplified a 1400 bp fragment from 7 *F. oxysporum* f. sp. *cubense* isolates (Bnt2, Mln1, Srg1, Bgl3, Bgl6 from Central Java, Lmp1 from Lampung Sumatera and Kjg1 from East Kalimantan). Thus, these 7 isolates belonged to tropical race 4 of *F. oxysporum* f. sp. *cubense*. One isolate (A13) with a weak reac-

tion to *nitM* tester representing 01213/16 was not shown to have positive result with specific TR4 primer. Amplification product were either not detected from the isolates that anastomosed with VCGs 0120 (GnK3, Wsb5 from Central Java), 0129 (Pjn4 from East Java). In addition, three odoratum isolates (Btu3, Tmg1, and Bnt1) also failed to anastomose with any of the testers used (Fig. 1).

The result of this study shows that race 4 strains of *F. oxysporum* f. sp. *cubense* are presented in Indonesia. Furthermore, it shows that tropical race 4 of *F. oxysporum* f. sp. *cubense* may be widely distributed in banana growing regions, as it was found in Central Java, Lampung Sumatera, and East Kalimantan attacking local banana cultivars such as Pisang ambon (AAA), raja (AAB), awak (ABB), kepok (ABB), kepok kerau (ABB), and raja nangka (AAB).

Acknowledgments

We wish to thank to General Director of Higher Education of Republic Indonesia and the Australian Centre for International Agricultural Research for funding this research. We also would like to thank Dr. Linda Smith from Queensland Department of Primary Industries Australia for supplying the *nitM* testers and to Prof. Paul Holford from The University of Western Sydney for reviewing this work and in preparing the manuscript.

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Fig. 1. PCR products from the genomic DNA of the isolates of *F. oxysporum* f. sp. *cubense* using the primer pair TR4-F2 and TR4-R1 on 1.5% agarose gel. Lane-1 1 Kb Marker; lanes 2–8 Bnt2, Mln1, Srg1, Bgl6, Bgl3, Lmp1, Kjg1 isolates; lane 9 A13 isolate; lane 10 Pjn4 isolate, lanes 11–12 GnK3, Wsb5 isolates; lanes 13–15 Btu3, Tmg1, Bnt1 isolates; lane 16 negative control.

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