FIRST REPORT OF STEWART'S WILT OF MAIZE CAUSED BY *PANTOEA STEWARTII* SUBSP. *STEWARTII* IN BOGOR DISTRICT, INDONESIA

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ABSTRACT

Stewart's bacterial wilt disease of maize caused by *Pantoea stewartii* subsp. *stewartii* is a disease newly found in Bogor district, Indonesia. This seed-borne pathogen has been reported to result in yield losses of 40-100%. The pathogen is listed as an Indonesian quarantine pest. The research objective was to survey the occurrence of the disease in Bogor District West Java maize growing areas and identification of the causative agent through morphology, physiology and molecular characters. The research was conducted in Plant Bacteriology Laboratory and Cikabayan green house facility, Department of Plant Protection, Bogor Agricultural University from March 2011 to August 2012. The Stewart's wilt disease incidence in Bogor area was 23.67 to 31.45%. Field symptoms on maize are pale or yellowish green leaf streaks, stunted growth at the younger stage, and leaf blight on older plants. The isolated bacteria grew on TSA medium showed bright yellow, shiny, convex, and 1-4 mm in diameter colonies. Tobacco plants injected with the bacterial suspensions showed hypersensitive reactions against 6 bacterial isolates. Four of these isolates were able to incite characteristic symptoms of Stewart's wilt on SD3 cultivar sweet corn plants that were used for bacterial pathogenicity tests. Biophysiological identification of the pathogen showed all bacterial isolates (BGR2, BGR4, BGR28 and BGR70) , were identified as *P. stewartii* subsp. *stewartii*. Further determination based on sequence analysis of 16S rRNA, *hrpS* and *cpsD* genes showed that all isolates were consistently found to be *P. stewartii* subsp. *stewartii.*

Key words: seed-borne bacterium, sweet corn, quarantine pest

INTRODUCTION

Maize is one of most important field crops and second main staple food after rice in Indonesia. Due to increasing demand of maize, which is not able to be fulfilled entirely by local growers, Indonesia has to import the commodity up to 1.4 million tonnes (Hosang *et al*., 2010). The maize importation by Indonesia in 2011 was valued at USD 1 billion, to supply human need and the animal food industry (Lakitan, 2012).

One impact of excessive importation of maize to Indonesia is the risk of introduction of new plant pathogens along with seeds, including *Pantoea stewartii* subsp. *stewartii* (Syn. *Erwinia stewartii*, a seed-borne bacterium causing maize Stewart's wilt disease. The pathogen can cause a devastating disease of maize and 40-100% yield losses (Freeman and Pataky, 2001). The Ministry of Agriculture Indonesia stated the bacterium is listed as an A1 quarantine pest and is prohibited to enter Indonesia.

Research on Stewart's wilt disease in Indonesia is still limited to early diagnosis primarily based on symptoms and simple bacterial culture or colony characterization, and therefore the disease causative agent is still unknown. This could be established through fulfillment of four criteria of Koch's Postulates (Janse, 2005) followed by characterization of causal agent by morphology, physiology and molecular characters of the Indonesian isolate found for the first time.

EPPO considers *P. stewartii* subsp. *stewartii* as an A2 quarantine pest (OEPP/EPPO, 1978) . Many countries ban its import unless it is certified to be free of *P. stewartii* subsp. *stewartii.* The important economic value due to this pathogens incited development of detection method both in conventional and modern technique. Wensing *et al,* 2010 developed the detection method based on MALDI-TOF MS analysis and specific PCR primers that were clearly distinguished *P. stewartii* from *Pantoea agglomerans*, *Pantoea dispersa*, and *Pantoea ananatis.* On the other hand, this bacteria is widely use as model for bacterial system for laboratory studies in plant pathology bacaused of this bacteriaappear to employ a minimal number of pathogenicity mechanism (Roper, 2011).

This research sought to survey the occurrence of the Stewart's wilt disease in Bogor district maize growing areas, confirmation of the causative agent through Koch's Postulates and its identification.

MATERIALS AND METHODS

Survey of Stewart's wilt disease of maize in Bogor district, West Java

Stewart's wilt disease of maize was observed in fields located in Bogor district, West Java and described based on the typical symptoms. The disease incidence was also recorded. Infected plant samples were taken back to the laboratory for further examinations.

Isolation of bacterium associated with Stewart's wilt disease

Infected plant samples were washed with running water, surface sterilized with 70% ethanol for 1 min, rinsed 3 times with sterile distilled water, and air dried on sterile paper towels. Bacterium were isolated following the method of Coplin *et al.* (2002). Symptomatic leaf samples were aseptically cut into 10 mm x 10 mm pieces and soaked in Tryptic Soya Broth (TSB) medium overnight to allow release of bacteria into the medium. The bacterial suspensions were then transferred to Tryptic Soya Agar (TSA) medium in Petri dish with 3 replications and incubated at 25-29 °C. Single bacterial colonies which were bright yellow, soft, shiny, and convex were separately isolated.

Characterization and identification of the causative agent of Stewart's wilt disease

a. Hypersensitive reaction tests on tobacco plants

Fresh bacterial culture at 10^8-10^9 cfu ml^{-1} was injected to the lower side of Virginia tobacco cultivar leaves to show hypersensitive reaction of the plant to the bacterium. Sterile water was used as the control. The death of tissues within 24 hours after inoculation indicated that the bacteria injected to the tobacco were plant pathogenic (Klement *et al*., 1990).

b. Pathogenicity tests of bacterial isolates on maize

Pathogenicity tests of bacterial isolates were carried out with SD3 sweet corn cultivar, following the method by Coplin and Kado (2001). Eight day after sawing of

sweet corn seedling (each bacterial isolate used 10 seedlings) were inoculated with bacteria with by two methods. The first method was by injecting the seedling pseudostems with bacteria at a density of 10^8 cfu ml^{-1} as much as 100 µl. The second method was by leaf whorl inoculation where bacterial suspensions at a density of 10^8 cfu ml^{-1} as much as 100 µl were placed on the curled leaf surfaces. Sterile distilled water was used as the controls for both methods of inoculation. The inoculated plants were kept in clear polythene cages at 90% RH and temperature range of 25-27°C for 24 to 48 hours. The early symptoms were expected to appear 3-5 days after inoculation including water soaking, chlorosis, necrosis, wilting and stunting.

c. Morphological and physiological characteristics of bacterium

The 24 hours old bacterial culture on TSA medium was used for morphology and physiology characterizations as recommended by Schaad *et al.* (2001) to distinguish the genus of an unknown bacterium. Gram determination was done with two procedures, i.e. Gram staining and 3% KOH test. In order to test the anaerobic growth, a basic medium containing peptone (2.0 g), NaCl (5.0 g), KH_2PO_4 (0.3 g) agar (3.0 g) and 3 ml of 1% bromthymol blue and 1% glucose solution per liter was prepared aseptically in test tubes. Bacterial isolates were added into two tubes of the medium. One of the them was overlaid with sterile liquid vaseline. Color change from blue to yellow indicated positive anaerobic growth (fermentative reaction) of the bacterium. YDC agar medium in petri dish was used to test for pigmentation.

The next step was to characterize the bacterium at species level. Acid production in carbohydrates was observed in Dye's C medium containing $NH_4H_2PO_4$ 0.5 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 5 g, yeast extract per liter medium overlaid with 10% w/v solution of maltose, mannose and glycerol which were previously filtrated through a filter of 0.22 µm (Corning, NY 148310). Acid production was indicated with color change to yellow. Bacterial motility was observed from the diffusely spread of the bacteria from the line of inoculation on agar medium. The ability of bacterium to grow at 37°C was observed on nutrient agar medium in petri dish incubated at 37°C for 3 days. The occurrence of bacterial colonies at the point of inoculation indicated positive growth.

d. Molecular identification of *Pantoea stewartii* subsp. *Stewartii*

- Identification based on 16S rRNA gene sequences. Bacterial genomic DNA was isolated using DNA extraction Kit (Geneaid). A prokaryotic universal primer pair 27F (5'-AGA GTT TGA TCM TGG CTC AG-3)/ 1492R (5'-AGA GTT TGA TCM TGG CTC AG-3') was used for the polymerase chain reaction (PCR) assay (Galkiewicz and Kellogg, 2008). P olymerase chain reaction (KAPA Taq Ready Mix, KAPA Biosystems) was at 25 µL in total, consisting of 20 pmol primer, genomic DNA template (100 pg). It was performed with initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, DNA synthesis at 72°C for 1.5 min, and the final extension at 72°C for 5 min. PCR products were separated on 1% agarose gel containing ethidium bromide at 75 V DC for 45 min. A ±1.5 kb DNA band target was expected to be visible under UV transilluminator. The PCR DNA product was sent directly for sequencing at First Base Laboratories Sdn Bhd Shah Alam Selangor, Malaysia. DNA sequences were aligned by using BLAST-N to determine the similarity of the isolates tested with homologous species available in a gene bank.

- Identification based on *hrpS* and *cpsD* gene sequences. DNA of bacterium isolated from symptomatic plants was PCR amplified by using HRP1d (5'-GCA ATT CCG CTC

ACC AC-3')/HRP3c (5'-GCG TAC GCA CTA ACT CC-3') and CPSL1(5'-CCT GTC AGT CTC GAA CC-3')/CPSR2c (5'-AAC ATC TCG CGG TAA CC-3') primer pairs targeting *hrpS* and *cpsD* genes respectively (Coplin *et al*., 2002). PCR (KAPA Taq Ready Mix, KAPA Biosystems) was done as above. with initial denaturation (94°C for 120 seconds), 25 cycles of denaturation (94°C for 20 seconds), annealing (58°C for 15 seconds for primer HRP and 55°C for 30 seconds primary CPS), elongation (72°C for 90 seconds), and the final extension (72°C for 5 seconds). PCR products were separated on 1% of agarose gel containing ethidium bromide at 75 V DC for 45 min. The expected DNA bands i.e. 0.9 kb of *hrpS* gene and 1.1 kb of *cpsD* gene were visualized under UV transilluminator. The amplification products were sequenced and analized for similarity of base sequence using BLAST program.

RESULTS AND DISCUSSION

Survey of of Stewart's wilt disease in Bogor District

The average percentage of the disease incidence in the field was 23.67% - 31.45% (Table 1). The survey at Bogor district was conducted at several sub-district and the characteristics Stewart's diseases was found at three sub-district i.e Sindang Barang, Dramaga and Cigombong.

Table 1. Incidence of Stewart's wilt disease in Districts of Bogor, Indonesia

Sub-District	Mean of Disease Incidence (%)
Sindang Barang	31.45
Dramaga	23.67
Cigombong	26.41

Symptoms such as changes in the color from pale green to yellow, linear lines with irregular or wavy margin parallel to the leaf veins, wilting and stunting of the crop were observed (Figure 1). Stunted plants were still capable of forming panicles but not ears. Stewart's wilt disease incidence was found in areas that were quite high and supported by current climatic conditions (rainy season, high humidity and warm temperature (28-33^{o}C). The symptoms were intermingled with downy mildew by *Peronosclerospora maydis*. According to OEPP/EPPO (2006), high temperatures greatly influence the severity of the disease in the field. The optimum temperature for growth of *Pantoea stewartii* subsp *stewartii* ranged between 18-29 ^{o}C (Pataky and Ikin, 2003). A continuous cultivation technique is advantageous for the bacterial pathogens as the host plants would always be available for them to complete their life cycle.

Isolation of bacterium associated with Stewart's wilt disease

The causal bacterium was extracted from the symptomatic leaves using TSA medium. Isolate characteristics on TSA media were cream-colored, bright yellow, shiny, round colonies, diameter 1-4 mm and convex in shape. *Pantoea stewartii* subsp. *stewartii* was reported to produce colonies with similar traits of yellow colour, smooth, glistening, and mucoid. The yellow colored and mucoid colonies are cultural characteristics of *Pantoeas* associated with the production of extracellular polysaccharides slime (EPS in media containing sugar). According to Mohammadi *et al*. (2012), *Pantoea stewartii* subsp. *stewartii* bacteria produced carotenoid pigment under the control of the EsaI/EsaR quorum-sensing system and most abundantly found in a high-cell-density situation. One characteristic of the bacterial isolate found in this research is shown on Figure 2.

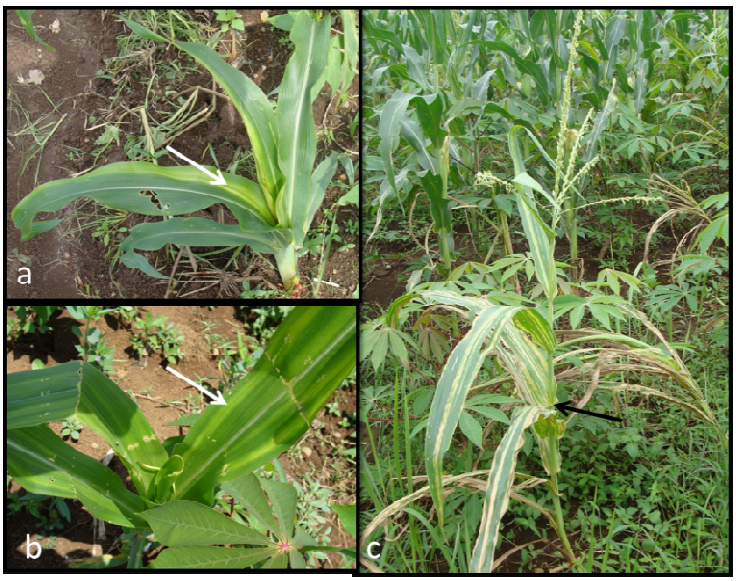

Fig. 1. Field symptoms of Stewart's wilt disease on maize (a and b) chlorotic and stunting at younger stage, (c) stunting at older stage.

Fig. 2. Colony of *Panthoea stewartii* subsp. *stewartii* isolate BGR28.

Characterization and identification of the causative agent of Stewart's wilt disease

a. Hypersensitive reaction tests on tobacco plants

In many plant-pathogen interactions, the resistance reaction is characterized by localized death of host cells in the region of infection (the hypersensitive response - HR). In these tests, the results of 6 isolates (BGR2, BGR4, BGR 10, BGR28, BGR 67, and BGR70) were positive on on tobacco leaves after 5-48 hours (data not shown). The positive HR response of these isolates were supported during their screening as potential pathogen. The isolates showed typical hypersensitive reactions on tobacco leaves inoculated by an injection infiltration technique. The leaf tissues were infiltrated with a high concentration of virulent strains which developed into

water soaked spots after 5 hours, discoloration occurred after 20 hours, becoming yellow and necrosis after 48 hours. The avirulent strains did not exhibit any necrotic symptom and the reaction behavior was delayed HR is characterized by rapid death of tobacco leaf tissues within a short period (24-48 hours) and is a rapid method to detect pathogenicity of bacteria (Lelliott and Stead, 1987).

b. **Pathogenicity tests of bacterial isolates on maize**

The results of pathogenicity tests were shown in Table 2. The incubation periods varied from 3-9 and 6-12 days after inoculation for the pseudostem and whorl inoculation method respectively. Incubation period of isolates BGR2, BGR4, BGR28 and BGR70 were faster than the others(BGR10 and BGR67). These isolates showed symptoms of water soaked lesion, chlorosis, yellow-green coloration on the surface of leaves, wilting and stunting of the crop. Other isolates produced symptoms observed as spots only. The results of inoculations indicated that the bacteria associated with Stewart wilt's disease could induce symptoms with similar characteristics as observed in the field.

Table 2. Disease symptoms from pathogenicity tests of bacterial isolates inoculated on sweet corn SD 3 cultivar.

Isolate	Pseudostem inoculation			Whorl inoculation		
	Incubation period (days)	Symptoms	Disease Incidence (%)	Incubation period (days)	Symptoms	Disease Incidence (%)
BGR2	4	Water soaked, chlorosis, necrosis and wilting	100	8	Water soaked, chlorosis, necrosis on one leaf	100
BGR4	3	Chlorosis, necrosis, wilting and stunting	100	8	Water soaked, chlorosis, necrosis on one leaf	100
BGR10	7	Watersoaked, chlorosis, necrosis at the point of inoculation	100	12	Water soaked, chlorosis, necrosis on one leaf	100
BGR28	3	Chlorosis, necrosis, wilting and stunting	100	6	Water soaked, chlorosis, necrosis on one leaf	100
BGR67	9	Water soaked, chlorosis, necrosis at the point of inoculation	100	11	Water soaked, chlorosis, necrosis on one leaf	100
BGR70	3	Water soaked, chlorosis, necrosis and wilting	100	8	Water soaked, chlorosis, necrosis on one leaf	100
Control	0	No symptoms	0	0	No symptoms	0

c. **Morphological and physiological characteristics of bacterium**

Biochemical tests results were shown in Table 3. All the isolates tested were negative for Gram reactions. Plant pathogenic bacteria that are gram-negative include *Erwinia, Pantoea, Ralstonia, Pseudomonas, Xanthomonas* and *Agrobacterium*. The anaerobic test is a key test for identification of the bacterium genera *Erwinia* and *Pantoea*. Yellow pigmentation on YDCA medium is produced by *Pantoea*. Four isolates were identified positive as *Pantoea* (BGR2, BGR4, BGR28, BGR70) based on characters as discribed by Schaad et al. (2001). The physiological charactereristics aimed to differentiate species indicated these isolates were similar to *Pantoea stewartii* subsp. *stewartii.* For accuracy, the physiological characteristics were subsequently confirmed by molecular identification. Since morphological and physiological characteristics (phenotypic) were dependent on the environmental factors and growing conditions of the bacterium, the results of molecular tests were considered more stable as they are not dependent on these (Suwanto, 1994).

Table 3. Biochemical tests results for selected bacterial isolates

Test	Bacterial isolate					
	1	**2**	**3**	**4**	**5**	**6**
Gram reaction	-	-	-	-	-	-
Anaerobic growth	+	+	-	+	-	+
Yellow pigment on YDC	+	+	nt	+	Nt	+
Motility	-	-	nt	-	Nt	-
Growth on 37 ºC	-	-	nt	-	Nt	-
Acid production on			nt		Nt	
- Maltose	-	-	nt	-	Nt	-
- Mannitol	+	+	nt	+	Nt	+
- Glycerol	-	-	nt	-	Nt	-

*1=BGR2, 2=BGR4, 3=BGR10, 4=BGR28, 5=BGR67, 6=BGR70, nt= not tested

d. **Molecular identification of Pantoea stewartii subsp stewartii**

Identification based on 16S rRNA gene sequences. Four isolates from Bogor BGR2, BGR4, BGR28 and BGR70 were analogous to partial sequences of the 16S rRNA gene *Pantoea stewartii* subsp. *stewartii* available in the GenBank data center (Table 4). These bacteria showed similarity levels up to 97% - 99% with the accession number AJ311838 (*Pantoea stewartii* subsp *stewartii* from USA; Illinois based on Coplin et al. 2002), AF373198 (*Pantoea stewartii* subsp. *stewartii* strain GSPB 2626 from USA based on Fessehaie et al. 2002) and NR044800 (*Pantoea stewartii* subsp. *stewartii* strain ATCC 8199 based on Kwon et al. 1997) respectively.

The bacteria can be identified with all 16S rRNA gene sequences available at the GenBank data center (Woese, 2006). The identified bacterium showed similarity levels ≥ 94% with those in GenBank (Clarridge, 2004). Gene of 16S rRNA has high accuracy as it was considered a major pathway for the biosynthesis of ribosomal protein in the evolutionary development of organisms (Weisburg et al. 1991).

Table 4. The similarityty of 16S rRNA gene sequence of bacterium causing Stewart's wilt disease with reference strain in GenBank

Isolate	Reference strain	Similarity (%)	Accession number
BGR2	*P. stewartii* subsp *stewartii*	98	AJ311838
BGR 4	*P. stewartii strain GSPB*	98	AF373198
BGR28	*P. stewartii strain GSPB*	97	AF373198
BGR 70	*P. stewartii* subsp *stewartii*	99	NR044800

Identification based on *hrpS* and *cpsD* gene sequences. The results of PCR amplification using specific primers HRP and CPS were shown in Figure 3. Four samples of bacterial DNA were detected using primers HRP and CPS, namely isolates BGR2, BGR4, BGR28, and BGR70 showing the position of amplicon at 0.9 kb and 1.1 kb respectively. These results were in line with previous report showing DNA fragment amplifications of Pantoea stewartii subspecies stewartii with specific primer HRP and CPS at 0.9 and 1.1 kb (Coplin et al. 2002).

Based on the sequencing of specific gene from three isolates BGR2, BGR4, and BGR28 showed 98-99% of similarity with *Pantoea stewartii* subsp. *stewartii hrpS* genes with accession numbers AF282857 (Frederick et al. 1993; Frederick et al. 2001). Isolates BGR2, BGR4, BGR28 and BGR70 showed about 89-99% similarity with *Pantoea stewartii* subsp. *stewartii cpsD* genes with accession numbers AF077292 (Coplin et al. 1996). According to Coplin and Cook (1990), two main groups of genes were important in the pathogenesis and virulence of *Pantoea stewartii* subsp. *stewartii*. Firstly, the *hrpS* gene cluster, which is encoded a type III secretion system. This gene is generally required for production of water soaking and the ability of bacteria to demonstrate hypersensitive reaction and pathogenicity (Lindgren 1997). Secondly, *cpsD* gene cluster is necessary to produce stewartan exopolysaccharide (EPS) contributing to symptoms of wilting and water soaking. This bacterium is not known to produce any extracellular toxins, degradative enzymes or plant growth hormones (Frederick et al. 2001).

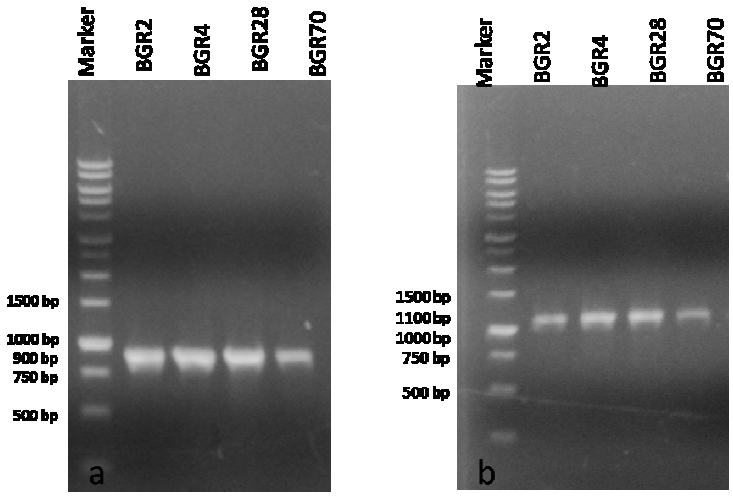

Fig. 3. Amplification of the specific gene of *Pantoea stewartii* subsp. *stewartii*. (a) *hrpS* gene using primers HRP1d and HRP3r, (b) *cpsD* gene using primers CPSL1 and CPSR2c. Products has been separated by 1% agarose gel electrophoresis. Marker 1 kb from Geneaid.

CONCLUSION

This study generated data to confirm the presence of *Pantoea stewartii* subsp. *stewartii* on cultivated maize in the Bogor district of West Java in Indonesia. Stewart's wilt disease symptoms were found in Bogor District of West Java, with a percent disease incidence ranging from 23.67 to 31.45%. Symptoms of the disease were the change of color to pale green or development of yellowish green leaf streaks, stunted plant growth in the younger stages, and occurrence of leaf blight on older plants. The growth of the bacterial isolates on TSA medium produced bright yellow, shiny, convex shaped colonies, 1-4 mm in diameter. Four isolates produced characteristic symptoms of Stewart's wilt on SD3 sweet corn cultivars when tested for pathogenecity. Based on 16S rRNA, *hrpS* and *cpsD* gene similarities, isolates BGR2, BGR4, BGR28, and BGR70 were identified as *Pantoea stewartii* subsp. *stewartii*.

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