

IDENTIFICATION OF BACTERIAL WILT AND LEAF BLIGHT DISEASE ON MAIZE (*Zea mays*) FOUND IN KEDIRI, INDONESIA

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ABSTRACT

Recently, a new bacterial disease of maize (*Zea mays*) was observed in Kediri, East Java, Indonesia. Infected plants showed wilt symptoms occasionally accompanied by leaf blight. This study aims to characterize the causal agent of bacterial wilt and leaf blight of maize observed in Kediri. Gram-negative, facultative anaerobic bacteria were isolated from the diseased tissues. All bacterial strains (KD1A, KB2A, KD1, KD4, KB1) gave positive result both in the hypersensitive response and pathogenicity assays. However, only KD1 and KB1 strains could be re-isolated from the diseased tissues. Based on several physiological and biochemical assays, the bacteria resembled *Pantoea agglomerans*. Moreover, the strains showed negative result on PCR amplification using HRP1d and HRP3r, a primer pair specific for detection of *P. stewartii* subsp. *stewartii*. Analysis of 16S rRNA gene sequence of KD1 and KB1 showed highest homology at 88% and 90%, respectively to *P. agglomerans* strain DSM 3493 (NR 0419781). The homology values were too low to conclude that the bacteria were similar to *P. agglomerans*. These results suggest that bacterial pathogens isolated from maize in Kediri were strains of *Pantoea* sp.

Keywords: maize, *Pantoea* sp., wilt, leaf blight

INTRODUCTION

Maize (*Zea mays*) is one of the most important grain crops in Indonesia. Maize is used as animal feed grain and raw materials for food industry (Akil and Dahlan, 2010). East Java

is the highest maize producer in Indonesia and became a center for maize seed industry. In 2011, the production of maize in East Java reached 5.443.705 tons yielded from 1.204.063 ha of production area (BPS, 2011).

Since 2009, a new disease of maize was reported from some maize production areas in Kediri, East Java. The disease was found on hybrid cultivar of maize planted in Kandangan and Pare sub-districts, varying from 1 to 8 %. The disease occurred when plants entered the generative phase with typical symptoms of wilt accompanied with blight. Wilted plants scattered in field with the disease incidence of 5-8%. The leaf blight symptom was brown with a flat edge. When the rotten stalks as well as blight leaves were cut, masses of bacteria observed came out of the cutting edge.

The occurrence of bacterial wilt on maize has been widely reported in several countries. The most widely known bacterial wilt and leaf blight disease of maize is Stewart's disease. The disease is caused by the pathogenic bacterium *Pantoea stewartii* subsp. *stewartii*, which enters the plant through feeding wounds of the insect vector, the maize flea beetle (*Chaetocnema pulicaria*) (Lipps *et al.*, 2003; Pataky, 2003).

There are other common bacterial wilt and leaf blight disease in maize i.e. *Clavibacter michiganensis* subsp. *nebraskensis*, the causal agent of Goss's bacterial wilt and blight (Smidt and Vidaver, 1986), *Pantoea agglomerans* causing leaf blight and vascular wilt of maize and sorghum in Mexico (Morales-Valenzuela *et al.*, 2007), and *Pantoea ananatis* associated with leaf spot disease which were reported either in South Africa (Goszczyńska *et al.*, 2007), Brazil (Paccola-Meirelles *et al.*, 2001), Mexico

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(Pérez-y-Terrón *et al.*, 2009), and Poland (Krawczyk *et al.*, 2010).

This study aims to determine the causal agent of bacterial wilt and leaf blight found in Kediri with several methods i.e. physiological assays, biochemical assays, molecular detection using specific primer for *P. stewartii* subsp. *stewartii* and homology of 16S rRNA gene sequences.

MATERIALS AND METHODS

Isolation and Identification of Bacterial Pathogen from Diseased Maize Plants

Bacterial pathogens were isolated from maize plant parts (leaves and stalks) showing wilt or blight symptoms collected from Kediri in July 2011. Small sections of stalk or leaf tissues with indicated symptoms were cut aseptically from the margins of lesions and macerated in 1 ml of sterile distilled water and incubated for 30 minutes. The suspensions were streaked onto nutrient agar, incubated at 28°C for 2 to 5 days. Each of grown bacterial colonies was purified and subjected to initial assays for the identification i.e. the Gram staining, Hugh-Leifson assay, pigment production on YDC, and hypersensitive reaction (HR) on tobacco.

Pathogenicity and Koch's Postulate Assays

Pathogenicity assay on maize plants was determined by inoculation of the suspension of bacterial isolates on maize cultivar P21 grown in pot. Each of bacterial suspension was prepared from 48 h bacterial culture grown on NA plates suspended in sterile distilled water to approximately 10^8 CFU/ml. Ten-days-old plants were inoculated with bacterial suspensions by using two methods: (i) infiltration into leaf tissues with a needle-less syringe and (ii) injection of suspension directly into maize plant stems. Inoculated plants were placed in a chamber and maintained in 90% relative humidity in room temperature for 2 days. After 2 days, the plants were moved to greenhouse. Bacteria were re-isolated from symptomatic leaves or stems tissues and further confirmed by observation of colony morphology on YDC, Gram staining, and Hugh-Leifson assay.

Physiological and Biochemical Assays

Strains were assayed as described by Schaad *et al.* (2000) i.e. growth at 37°C; nitrate

reduction; gelatin liquefaction; indole production; motility; utilization of citrate, malonate and tartrate; and acid production from glycerol, lactose, maltose, mannitol, sorbitol, meso inositol, cellobiose and sucrose.

DNA Isolation

Genomic DNA of bacteria was isolated using alkali lysis method (Ausubel *et al.*, 1996) with minor modification. The 24 hours cultured bacterial cells were harvested by centrifugation at 10.000 g for 5 minutes and re-suspended in 525 µl TE buffer. Bacterial cells were then disrupted by adding 60 µl of 20% SDS and Proteinase K (200 µg/ml), and incubated at 37°C for 60 minutes. The suspension was then mixed with 100 µl of 5N NaCl and 80 µl of CTAB and incubated at 65°C for 30 minutes. 750 µl Chloroform Isoamyl Alcohol (CIAA) were added in the suspension, mixed thoroughly and separated by centrifugation at 11.000 g for 10 minutes. Supernatant was transferred into new tubes and an equal volume of Phenol Chloroform Isoamyl-alcohol (PCI) was added, mixed thoroughly and separated by centrifugation at 11.000 g for 10 minutes. The DNA from the supernatant was precipitated using an equal volume of isopropanol and then centrifugated at 12.000 g for 2 minutes. DNA precipitate was further washed with 70% ethanol, re-centrifuged at 12.000 g for 2 minutes, dried at room temperature and re-suspended with 30 µl TE buffer containing RNAase.

PCR Assay

For the determination of pathogenic *Pantoea*, specific primers HRP1d (5'-GCACTCATTCCGACCAC-3') and HRP3r (5'-CGGCATACCTAACTCC-3') designed for the detection of *P. stewartii* subsp. *stewartii* (Coplin *et al.*, 2002) were used in this assay. PCR reaction was performed in 20 µL volumes using 2 µL of template DNA, 10 µL Go Taq® Green Master Mix 2X from Promega, 2 µL of 100 pmol of each primer, and 4 µL double distillate water (DDW). The condition of PCR amplification involved: 1 cycle at 95°C for 1 min, 55 cycles of: 30 s at 95°C, 30 s at 55°C, 60 s at 72°C, and 1 cycle of post extension at 72°C for 3 min. The PCR product was separated on 1.5% agarose gel at 1.5 V/cm, stained in ethidium bromide solution (0.5 µg/mL in TAE buffer) for 15 min,

and subjected to Gel-Doc observation. The size of the expected amplicon was: 0.9 kb.

Sequence Analysis

The partial 16S rRNA gene fragment was amplified using the universal primers fd1(5'-AGAGTTGATCCTGGCTCAG-3') and rd1(5'-AAGGAGGTGATCCAGCCGCA-3') (Weisburg *et al.*, 1991). DNA was amplified in 25 µl reaction volumes containing 4 µl template DNA, 12,5 µl Go Taq® Green Master Mix 2X from Promega, 2,5 µL of 100 pmol of each primer, and 3,5 µL DDW. The condition of PCR amplification involved 1 cycle at 95°C for 3 min; 40 cycles at 95°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min; post extension at 72°C for 3 min. For the confirmation, the PCR product was separated on 1.5% agarose gel at 1.5 V/cm. The DNA fragment was stained in ethidium bromide solution (0.5 µg/mL in TAE buffer) for 15 min, and subjected to Gel-Doc observation. The size of the expected amplicon was 1,5 kb.

The amplified PCR products were purified using ethanol/EDTA precipitation method. The sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequences were analyzed using Sequencing Analysis Software v5.31 (Applied Biosystems, USA). The GenBank/EMBL

database was used for homology search using the BLASTN program available in National Center for Biotechnology Information (NCBI) website: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

RESULTS AND DISCUSSION

In the field, the wilt symptoms were found on 70-day-old maize plants. The wilted maize plants released bacterial mass from the cutting of rotten stalk. The leaf blight with flat edge symptoms did not always occur on the wilt plants (Figure 1).

Isolation of Bacterial Pathogen and Hypersensitive Assay

Five strains KD1A, KB2A, KD1, KD4, and KB1 were isolated from leaves and stalks showing disease symptoms. The bacteria were able to grow on NA medium in less than 24 hours at 27-30°C. Colonies were circular, 1-2 mm in diameter, somewhat convex with the flat edges. All strains were Gram-negative rod, facultative an-aerobic and showed yellow colonies in YDC medium, suggesting that all bacterial strains are include in the genus of *Pantoea*. All bacterial strains produced HR in tobacco leaves 48 hour after infiltration (data not shown).



Figure 1. Disease symptoms on maize observed in the field
A. wilted plants; B. leaf blight C. rotten stalk

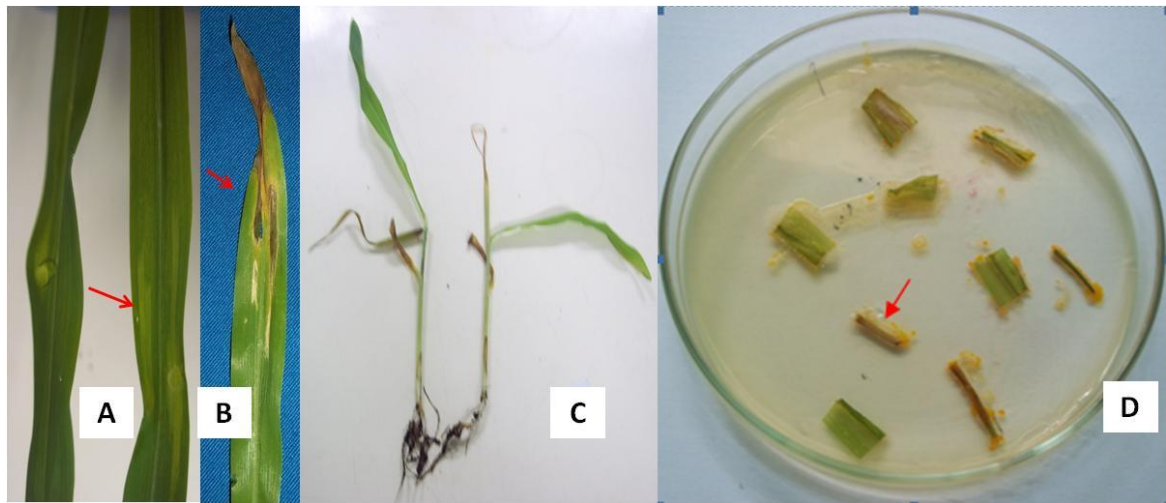


Figure 2. Symptoms on the pathogenicity test

A. water soak appeared on leaf B. leaf blight C. wilted plants D. bacterial colony from re-isolated plant parts

Pathogenicity Test

When leaves of 10-day-old maize seedlings were infiltrated with each strain of the bacterial suspension, water soaked symptom emerged in 2 days after inoculation. The symptoms changed into blight with irregular edges 4 days after inoculation. When the bacterial suspensions were injected into the stems of 10-day-old maize plants, at 5 days after inoculation, only KD1, KB1, and KB2A strains could produce wilt symptoms similar to those observed in the field. Maize plants inoculated with water did not develop any symptoms. Only plants that were inoculated with strain KD1 and KB1 had successfully been re-isolated from the symptom to prove the Koch's postulate (Figure 2). Bacterial colonies recovered from diseased symptoms were yellow, Gram negative, and facultative an-aerobic.

Physiological and Biochemical Characteristics

Physiological and biochemical assays (Table 1) showed that all bacterial strains had

characteristic resembling *P. agglomerans* described by Schaad *et al.* (2000) although variations occurred in several assays. The similar characteristics of bacterial strains to *P. agglomerans* i.e. able to grow at 37°C, motile, produce indole, utilize citrate and malonate, and produce acid from lactose, mannitol, cellobiose and sucrose. Bacterial strains showed varied responses in other assays compared to those of *P. agglomerans* (Table 1).

Detection Using Species Specific Primers HRP1d and HRP3r for *P. stewartii* subsp *stewartii*

Electrophoresis of PCR amplification product from genomic DNA of bacterial strains using primers HRP1d and HRP3r indicated that no 0,9 kb amplicon was detected on all bacterial strains compared with that of DM5262 strain used as positive control. The results showed that all tested bacterial strains were not *P. stewartii* subsp. *stewartii* (Figure 3)

Table 1. Physiological and biochemical characteristics of the bacterial strains isolated from maize, and published characteristic of *Pantoea stewartii* subsp. *stewartii*, *Pantoea ananatis* and *Pantoea agglomerans* (Schaad *et al.*, 2000)

Characters	<i>P.stewartii</i> subsp. <i>stewartii</i>	<i>P.ananatis</i>	<i>P. agglomerans</i> (herbicola strains)	KD1A	KB2A	KD1	KD4	KB1
Growth at 37°C	-	+	+	+	+	+	+	+
Yellow pigmen on YDC	+	+	+	+	+	+	+	+
Indole production	-	+	V	+	+	-	-	+
Nitrate reduction	-	V	+	-	-	+	-	-
Gelatin liquification	-	+	+	+	+	-	+	+
Motility	-	+	+	+	+	+	+	+
Utilization of:								
* Citrate	-	+	+	+	+	+	+	+
* Malonate	-	-	+	+	+	+	+	+
* Tartrate	-	+	-	-	-	+	-	-
Acid production from:								
* Gliserol	-	+	-	-	-	+	+	-
* Lactose	-	+	V	+	+	+	+	-
* Mannitol	+	+	+	+	+	+	+	+
* Sorbitol	V	+	-	-	-	-	+	-
* Cellobiose	-	+	V	-	-	+	-	+
* Maltose	-	+	+	+	+	+	+	+
* Meso inositol	-	+	-	+	+	-	+	+
* Sucrose	+	+	+	+	+	+	+	+

Remarks: V, between 21-79% of strains positive

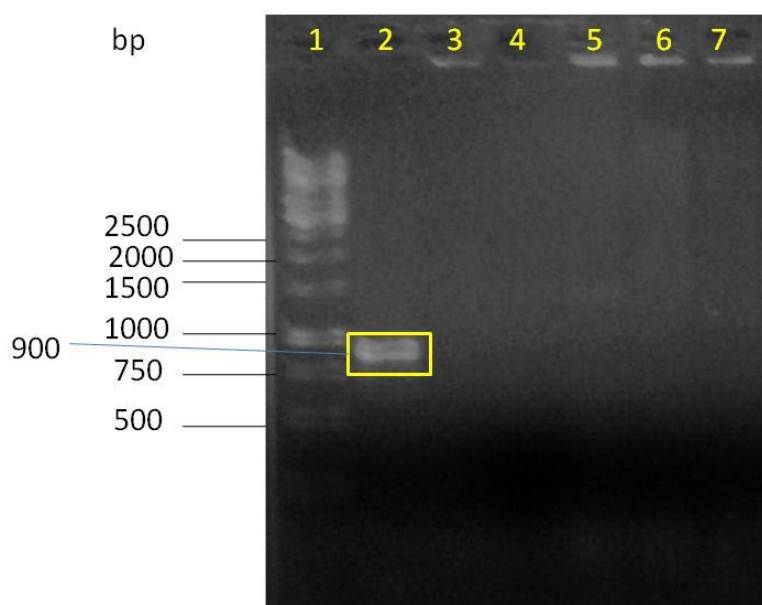


Figure 3. Visualization of PCR amplified amplicon of bacterial genomic DNA using HRP1d and HRP3r primers; 1. DNA 1 kb ladder; 2. DM5262; 3. KD1A; 4. KB2A; 5. KD1; 6. KD4; 7. KB1

Table 2. The result of BLASTN homology search

Strain	Query ID	BLAST result	Max score	Query coverage	E value	Max identity
KD1	lcl 10743	<i>P. agglomerans</i> strain DSM 3493(NR 041978.1))	1578	93%	0	88%
KB1	lcl 15531	<i>P. agglomerans</i> strain DSM 3493(NR 041978.1))	1801	100%	0	90%

Partial 16S rRNA Gene Sequence Analysis

BLASTN searching of partial 16S rRNA gene sequences showed that KD1 and KB1 had the highest homology at 88% and 90%, respectively to *P. agglomerans* strain DSM 3493 (NR 0419781) (Table 2). These values were too low to conclude that both strains were *P. agglomerans*.

Based on physiological, biochemical and molecular assays described above, it suggests that the bacterial wilt and leaf blight disease on maize found in Kediri was not caused by *P. stewartii* subsp. *stewartii*. Physiological and biochemical characters showed that the bacteria resembled *P. agglomerans*. However, the BLASTN homology using partial 16S rRNA gene sequence amplified from genomic DNA of those bacterial strains showed low similarity to *P. agglomerans*. Thus, we proposed these bacteria as strains of *Pantoea* sp. *Pantoea* sp. are members of the *Enterobacteriaceae* family that have traditionally been regarded as saprophytes with a cosmopolitan distribution. The typical the genus *Pantoea* comprises Gram-negative rod, facultative an-aerobic and showed yellow colonies in YDC medium. Recent taxonomic studies have separated the genus into groups of biocontrol, plant, and clinical strains (Rezzonico *et al.*, 2009; 2010). It was reported that strains of *Pantoea* sp. that resembles *P. agglomerans* causes stunting, with vertical crack symptoms at the first internode of maize in the Northwest and Mpumalanga Provinces of South Africa (Goszczyńska *et al.* 2007). Our results suggest that variant of strains of *Pantoea* sp could probably occur in various places, and to our case, these strains were pathogenic to maize. Further research on the disease is necessary to develop strategies to manage the disease.

CONCLUSIONS

Based on physiological, biochemical and molecular assays, the causal agent of wilt and leaf blight disease on maize found in Kediri were strains of *Pantoea* sp. Further research on the

disease is necessary to develop strategies to manage the disease.

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